NOVEL COLLAGEN-BASED SCAFFOLDS FOR HOLLOW ORGAN REGENERATION

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Luuk Versteegden

Voor mijn grootste voorbeeld.

NOVEL COLLAGEN-BASED SCAFFOLDS FOR HOLLOW ORGAN REGENERATION

DOCTORAL THESIS

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Dedicated to my greatest example.

CONTENTS

THESIS OBJECTIVES AND OUTLINE OF THESIS

This thesis aimed at developing new techniques, which can induce new properties to- or improve the properties of collagen-based materials for Tissue Engineering and Regenerative Medicine and subsequently the quality and precision with which the scaffolds can be made.

In previous literature, scaffolds for repair of hollow organs have been widely described for smaller diameter organs such as blood vessels and the gastrointestinal tract of smaller animals. This thesis focuses on the techniques for developing large diameter hollow scaffolds for possible use in the repair of organs in the gastrointestinal and urogenital tract. **Chapter 1** gives an overview of the current literature regarding the array of different biomaterials and subsequent processing techniques available to the tissue engineering and Regenerative Medicine community. **Chapter 2** reviews and scrutinizes the literature regarding the current status and future prospective of Regenerative Medicine regarding the respiratory tract. **Chapter 3** presents an investigation pertaining to the biocompatibility and subsequent *in vivo* vascularization of three different large-diameter tubular collagen-based scaffolds. In **chapter 4** the development of large-diameter tubular collagen-based scaffolds are continued and the subsequent *in vivo* evaluation for use as a urostomy is described. **Chapter 5** describes a novel and highly adjustable method for producing large and small-diameter tubular collagen-based scaffolds with a defined collagen fiber orientation by method of counter-rotating cone extrusion. **Chapter 6** postulates a novel strategy for the possible replacement of a bladder utilizing a seamless spherical hollow collagen scaffold with integrated anastomotic sites for the urethers and urethra. **Chapter 7** presents a novel technique to induce elastic-like characteristics in collagen scaffolds using a combination of physical restriction and chemical crosslinking. Finally, in **chapter 8** a summary of this thesis and future prospects are given regarding the results found over the course of this study.

CHAPTER 01

EXTRACELLULAR MATRIX DERIVED BIOMATERIALS:

MOLECULARLY DEFINED INGREDIENTS AND PROCESSING TECHNIQUES

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1 INTRODUCTION TO TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Tissue engineering (TE) and Regenerative Medicine (RM) are new rapidly growing disciplines in medical science. The need for TE and RM partially originates from the chronic shortage of auto- or heterologous tissues and organs for transplantation in cases of e.g., trauma, tumors and general necrosis. Often a distinction is made between soft and hard tissues where hard tissues refer mainly to bone, teeth and cartilage. This thesis will refer to soft tissue engineering from now on, or unless stated otherwise. The drawback of traditional allo- and xenogenic organ or tissue transplantations is that, due to the genetic variation of species and individuals, it is subject to immunological compatibility complications which only increases the severity of the shortage.[1] TE and RM are based on combining knowledge gathered from the fields of molecular life sciences, cellular biology, biomedical engineering, (bio) materials science, reconstructive surgery and transplantation biology, to develop (bio) medical devices and treatments which aid in the repair or replacement of damaged tissues and organs.[2, 3] More specifically, these biomedical devices can either be the supplementation of vital cells, extracellular matrix, biomolecules or a combination thereof, to the damaged site. On the contrary of popular belief, the terms tissue engineering and Regenerative Medicine cannot be used interchangeably. The term 'tissue engineering' was officially introduced in during the 1987 meeting of the National Science Foundation in Washington D.C. after an increasing amount of scientists were conducting research on controlled tissue repair.[4] The current (2014) definition being; "A substance that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components

of living systems, the course of any therapeutic or diagnostic procedure", according to the journal; Biomaterials. The term 'Regenerative Medicine' was first found in an article regarding hospital administration by Leland Kaiser.[5] The fact that he connected the Regenerative Medicine with hospital administration is not surprising because Regenerative Medicine actually is a connective term which encompasses several fields of medical research and (pre-)clinical applications, including tissue engineering. In 2007, Daar and Greenwood managed to propose an accurate yet reasonably concise definition; "Regenerative Medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause, including congenital defects, disease, trauma and ageing. It uses a combination of several converging technological approaches, both existing and newly emerging, that moves it beyond traditional transplantation and replacement therapies. The approaches often stimulate and support the body's own self-healing capacity. These approaches may include, but are not limited to, the use of soluble molecules, gene therapy, stem and progenitor cell therapy, tissue engineering and the reprogramming of cell and tissue types".[6] In 2008, Mason and Dunnill shortened the definition to "Regenerative Medicine replaces or regenerates human cells, tissue or organs to restore or establish normal function".[7] Figure 1 gives a schematic overview of the various fields of research contributing to Regenerative Medicine. This chapter aims to provide a fundamental and tutorial overview of the ECM and its components and highlights its essential role in RM of (soft) tissues. Subsequently, the current techniques used to process the ECM components into scaffolds for different applications are summarized and comprehensively reviewed.

2. TE & RM STRATEGIES

Reconstructive surgery dates back to the 5th century BC, wherein Sushruta described over 300 surgical procedures in his book; "Sushruta Samhita".[8, 9] Xeno- and allogenic tissue transplantations date as far back as early as the 16th century.[10, 11] Between 1890 and 1920 many important findings were made

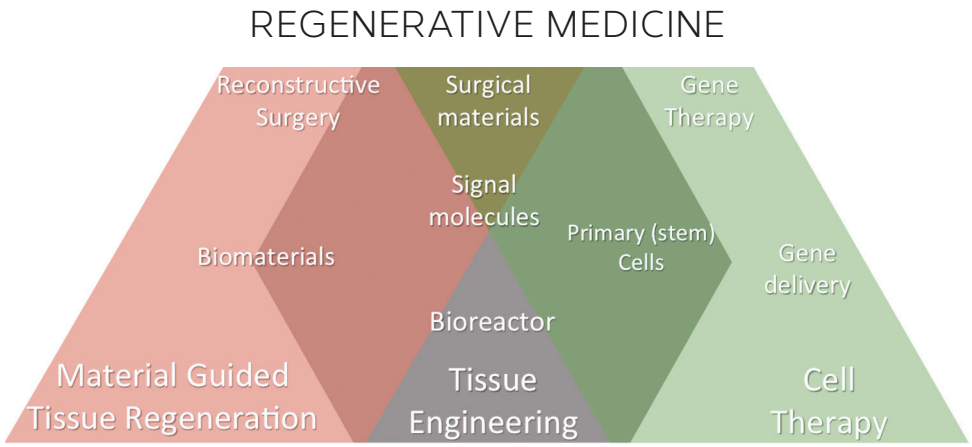

FIGURE 1: OVERLAY CHART GIVING AN OVERVIEW OF THE FIELD OF REGENERATIVE MEDICINE.

which made *in vitro* cell culture possible.[12-15] Tissue engineering's path of emergence coincided with that of general cell culture and its potential was first recognized in the late 1930s.[16] In era of World War I and II large amount of experience and data was gathered by experimental surgical procedures that were performed on disfigured soldiers. For example, during facial and cranial reconstruction the simplest form of tissue regeneration was frequently used in the form of skin and bone autografts. It is widely accepted that this has laid a basis for many modern reconstructive surgical techniques.[17, 18] Although these types of transplantations are not considered to be genuine TE, it has lead to one of the most important strategies, namely, the development materials which mimic the extracellular matrix (ECM). The ECM has many functions e.g., providing structural support for cells to reside, determining the mechanical properties of the tissues, providing mechanical signals to allow for cellular response and acting as a growth factor reservoir.[19] The strategy is based on stimulating the body's natural wound healing mechanism by implanting natural or synthetic ECM materials, with or without cells, into a defect. The ECM essentially acts as a template in the early stage of regeneration for cells to adhere or migrate to, proliferate and differentiate. Ideally, this hinders the formation of scar tissue and subsequently stimulates formation of new functional tissue, thus regenerating the defect.[20] These extracellular matrices, also referred to as biomatrices or scaffolds, can be classified into two groups: the decellularized tissues[21, 22] or molecularly defined constructs comprised of natural and/or synthetic biomaterials.[22, 23] Some proposed therapies are based on cells only but have only limited application in e.g., HIV, leukemia, heart failure, insulin-dependent diabetes, spinal cord injury, Parkinson's and Alzheimer's diseases. The supplementation of these cells frequently do not need a extra cellular matrix for proliferation and structural support, but rather only as a medium to keep the cells in the right place.

3. THE EXTRACELLULAR MATRIX

3.1 ROLE OF THE EXTRACELLULAR MATRIX (ECM)

In multicellular organisms the same set of rules biological rules generally apply. Cells can specialize in certain tasks, which in turn allow them to be more efficient. Due to the evolutionary process, cells form tissues in larger organisms, which in turn form organs that carry out a special function.[24] The cells are genetically identical, however, their gene expression depends on their function. The expression of specific genes leads them to produce biomolecules like proteins, carbohydrates, fats and metabolites. The cells are surrounded and subsequently held together by the ECM, which is produced by the cells themselves. The ECM is a dynamic and multifarious network that surrounds cells, providing structural and mechanical support in all tissues, mediating diverse biological processes that are crucial for supporting tissue formation and function, and playing an important role in wound healing. Cells adhere to the ECM via receptors (e.g., integrins) to maintain tissue architecture *in vivo*. ECMs can therefore regulate cellular functions by directly activating intracellular signaling pathways.[25] Depending on the function of the organ, the composition of cells and ECM differ tremendously. The composition of the ECM is specialized in different tissues in order to employ tissue specific cellular functions. For example, organs that have loadbearing functions like bone, cartilage, tendons and ligaments often have ECM's which are either rich in collagen, minerals or a combination thereof.[26] Organs with functions that require repetitive motions often contain collagen and elastin as the main structural component of the ECM like in the skin, lung and diaphragm.[27] In different tissues different ECM structures can be found, where for example the epithelial and dermal layer have dissimilar compositions. The complexity increases when taking into account that the composition of ECM constantly changes along with developmental stages of an organism[28] and also through pathological conditions, like fibrosis after trauma.[29]

3.2 ECM CONSTITUENTS

The ECM mainly consists of collagens, elastin, proteoglycans, laminins, fibronectins and glycosaminoglycans. Collagen provides tissues with essential tensile strength, enabling resistance to plastic deformation and rupture.[30] Type I collagen is the most abundant collagen and is present as a fibrillar protein, often accompanied by other collagen types like II, III, IV and V.[31] Elastin on the other hand, provides tissues the properties of extensibility and reversible recoil, enabling tissues to withstand repetitive mechanical stress.[32] The ECM is filled with a viscous interstitial fluid rich in proteoglycans (PGs) and other glycoproteins, containing a complex mixture of proteases, growth factors, cytokines and other effector molecules. Fibronectins can be found as a structural insoluble glycoprotein or soluble plasma protein, and contains binding domains that can immobilize other ECM components. Laminins are large trimeric glycoproteins that can be found in basement membranes. Laminins also have the ability to bind ECM components and can interact with cells with specific domains. Depending on the organ/tissue function different collagens can be found with different combinations of elastin, laminins, fibronectins and PGs. PGs consist of a core protein with a varying amount of glycosaminoglycans (GAGs) chains attached to them. GAGs are long, linear and strongly negatively charged polysaccharides, containing repeating disaccharides. GAGs are an important part of the ECM and govern countless functions from cell growth and differentiation to wound healing and cell protection. In combination with the fibrous proteins, PGs and GAGs play an important role in the maintenance of optimal visco-elastic characteristics, compressive stiffness and tissue hydration by sequestering water.[33] The main GAGs are chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate, keratan sulfate and hyaluronate (a GAG not bound to a core protein). GAGs also sequester a wide range of growth factors, consequently acting as a reservoir. Changes in physiological conditions, like trauma or infection, can trigger protease activities that cause local release of such depots allowing the rapid and local growth factor-mediated activation of cellular functions.[34] These components will be further elaborated next.

4. MOLECULARLY DEFINED BIOMATERIALS

One of the two fundamentally different strategies to design the scaffolds follows the "bottom-up" philosophy, making use of molecularly-defined biomaterials.[19,35] This section will discuss molecularly-defined biomaterials produced from naturally occurring ECM components. In contrast to the decellularization strategy, components of molecularly defined scaffolds are individually purified and can subsequently be used in combination with a variety of processing methods. Therefore substituting the use of cadaver tissue can improve standardization, quality and safety issues that inherently accompany decellularization strategies (see section 5.6). Advantages of molecularly defined materials are related to increased versatility, controllability, reproducibility and safety of the products. The potential and subsequent advantages of using defined biomaterials have been demonstrated in numerous studies.[35-39] Typically, depending on the type of molecularly defined biomaterials, they can be processed into different types of scaffolds (discussed in section 5). Biological and mechanical prerequisites of the defined biomaterials in question are **(i)** to support and deliver cells, **(ii)** to induce and differentiate tissue growth, **(iii)** to function as cell adhesion substrate, and **(iv)** to stimulate cellular response. Other important materials properties include **(v)** formation of a wound-healing barrier, **(vi)** biocompatibility and controlled (non)-biodegradability, **(vii)** relative ease of processability and malleability into desired shapes, **(viii)** mechanical strength and dimensional stability and **(ix)** sterilizability.[40] The used materials can either be of natural or synthetic origin. In this chapter only materials found in the extracellular matrices of natural origin will be discussed.

4.1 MAMMALIAN ECM-BASED MATERIALS

Biomaterials isolated from natural sources have been widely used in the RM arena. Early research focused on proteins isolated from the human ECM and their roles in wound healing. From the primary amino acid sequence up to the tertiary/quaternary

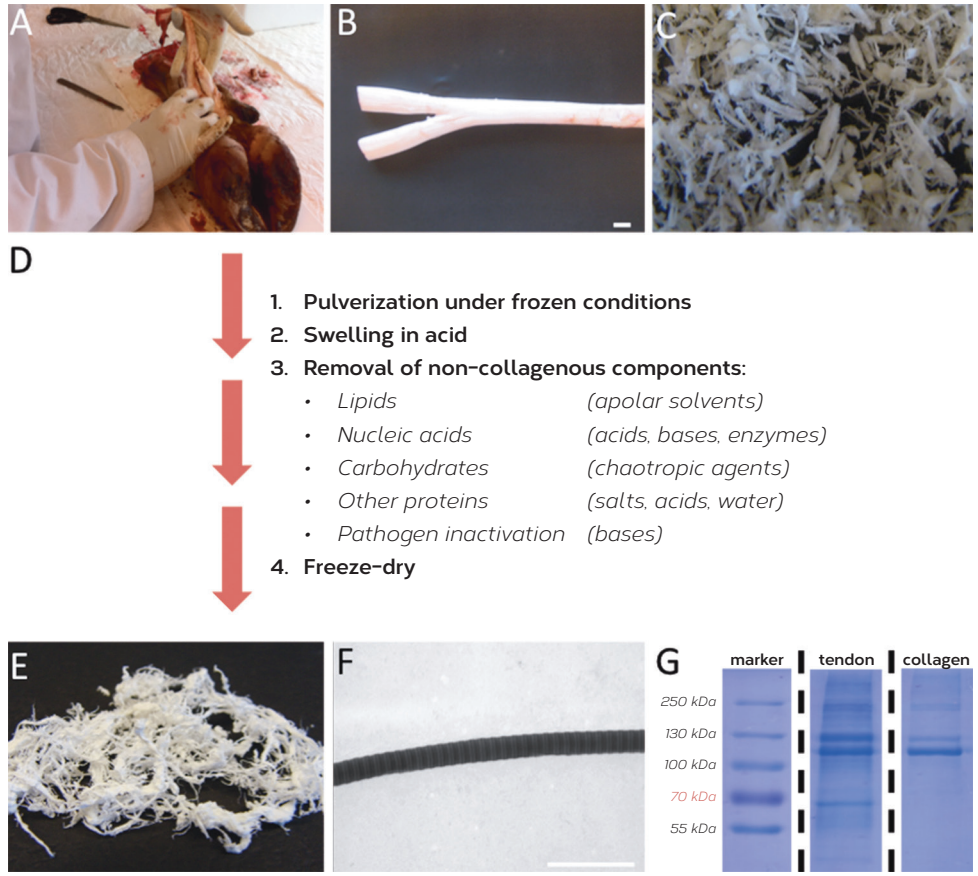

FIGURE 2: ISOLATION OF COLLAGEN AS A BIOMATERIAL.

A) Achilles tendons are removed from bovine legs and **B)** subsequently freed from macroscopical impurities such as fatty membranes and blood, bar represents 1 cm. **C)** The cleaned tendon is pulverized under frozen conditions followed by **D)** washing steps with acids, bases, chaotropic agents, salts and organic solvents to remove non-collagenous components. **E)** This procedure results in purified collagen fibrils as a basic material. Several quality assurance steps can be taken; **F)** transmission (TEM) to visualize the quarter staggered array structure of the collagen fibrils, bar represents 500 nm and **G)** SDS-PAGE to assess protein impurities in the final product. The characteristic collagen bands can be observed and does not contain other proteins found in the raw material (tendon).

structure of extracellular matrix proteins, the degree of conservation is generally high throughout primates and ungulates. A high degree of homology in structure might theoretically reduce the chance of an unsolicited host immune response. Therefore, many attempts have been made to identify ubiquitous sources of the ECM specific component in question, in genetically related mammalian species. Next to ECM proteins, polysaccharides (e.g., GAGs) are also subject to substantial research interest due to their versatility and roles they play in the ECM. In general, the organs of interest are retrieved from a standardized source after which the surface area is increased (pulverization) to facilitate future processing steps. The material is then subjected to an array of carefully selected chemicals and enzymes to remove the fractions that are not of interest. After isolation, the ECM component is subjected to tests in order to determine its purity and (near) native state. This central scheme is found in most processes used to obtain ECM components (figure 2). Proteins and GAGs are the main component of the human ECM. However, many other natural materials have been isolated from other non-mammalian sources like for example from plants or crustaceans. Even though these isolated materials are not native to the human physiology, some are biocompatible and can mimic certain functions of the natural ECM. In this section an overview will be given of the different available natural materials currently being used in TE&RM research.

4.1.1 COLLAGEN

Collagen is the most abundant protein in man and most other vertebrates. It is a major structural polymer which can be found throughout the body providing structural integrity and rigidity in tissues like tendons, cartilage and skin.[41] Along with calcium, collagen is important for strength and structure in bones and teeth. So far more than 28 genetically distinct types of collagen have been found, of which type I collagen most abundant.[42] On a cellular level, type I collagen mainly acts as a structural component but it also mediates biological functions like cell binding, migration, growth and chemotaxis.[43] Type II is predominantly found in cartilage whereas type III can be found in more elastic tissues like the skin. Type IV is a universal component of the basement membrane, a thin sheet of specialized ECM upon which a large number of cell types (epithelium, endothelium, muscle cells) rest and which plays an essential role in cell adhesion. Collagen-based biomaterials have been widely used for tissue engineering applications for a number of reasons, including biocompatibility, biodegradability, low immunogenicity and low antigenicity.[44]

4.1.1.1 COLLAGEN BIOSYNTHESIS AND STRUCTURE

Figure 3 shows a schematic representation of the collagen biosynthesis. The consensus sequence (glycine-X-Y)$_n$ gives rise to α-chains, the peptide subunit, of the fibril-forming collagen helix (types I, II, III, V, and XI).[45] The X and Y can be any amino acid however; the positions are frequently occupied by proline and hydroxyproline, respectively. The hydroxyproline and proline provide rigidity due to their unusual ring-like structure in the peptide backbone and force the peptide chain into a left-handed helix. Three identical α-chains (homotrimers in the case of e.g. collagen types II and III) or two or more different α-chains (heterotrimers in the case of e.g. collagen types I, and IV) are twisted together to form a right-handed triple helix. This triple helix is also known as tropocollagen and is, like DNA, stabilized by hydrogen bonds.[45] The smallest amino acid (glycine) allows tight packing of the three α-chains and provides flexibility to the peptide backbone. Tropocollagen is the basic molecular unit of "collagen" with a general molecular weight of about 300 kDa, length of 280 nm and width of 1.5 nm.[46] At the ends of the collagen molecule, there are areas characterized by lack of hydroxyproline and proline and subsequent lack of triple helical structure. These 'frayed' ends of the collagen molecule are also referred to as the telopeptide regions (at both the N- and C-terminal ends). The telopeptide region is rich in lysine and hydroxylysine residues and has four main functions, namely: i) the stabilization of the molecule via intramolecular crosslinking, ii) formation and stabilization of collagen fibrils via intermolecular crosslinking, iii) providing crosslinking points with other extracellular matrix proteins and iv) the formation of "sticky ends" by disulfide bonds to aid self-assemebly.[47] The tropocollagen molecules assemble in a quarter-staggered fibril array, so that each molecule overlaps approximately one-fourth with its neighboring molecule (67 nm). This process repeats itself until a fibril of a certain dimension is formed. This phenomenon can be demonstrated under the transmission electron microscope, where a characteristic banding pattern of alternating light and dark striation can be seen. This characteristic banding pattern has a periodicity of

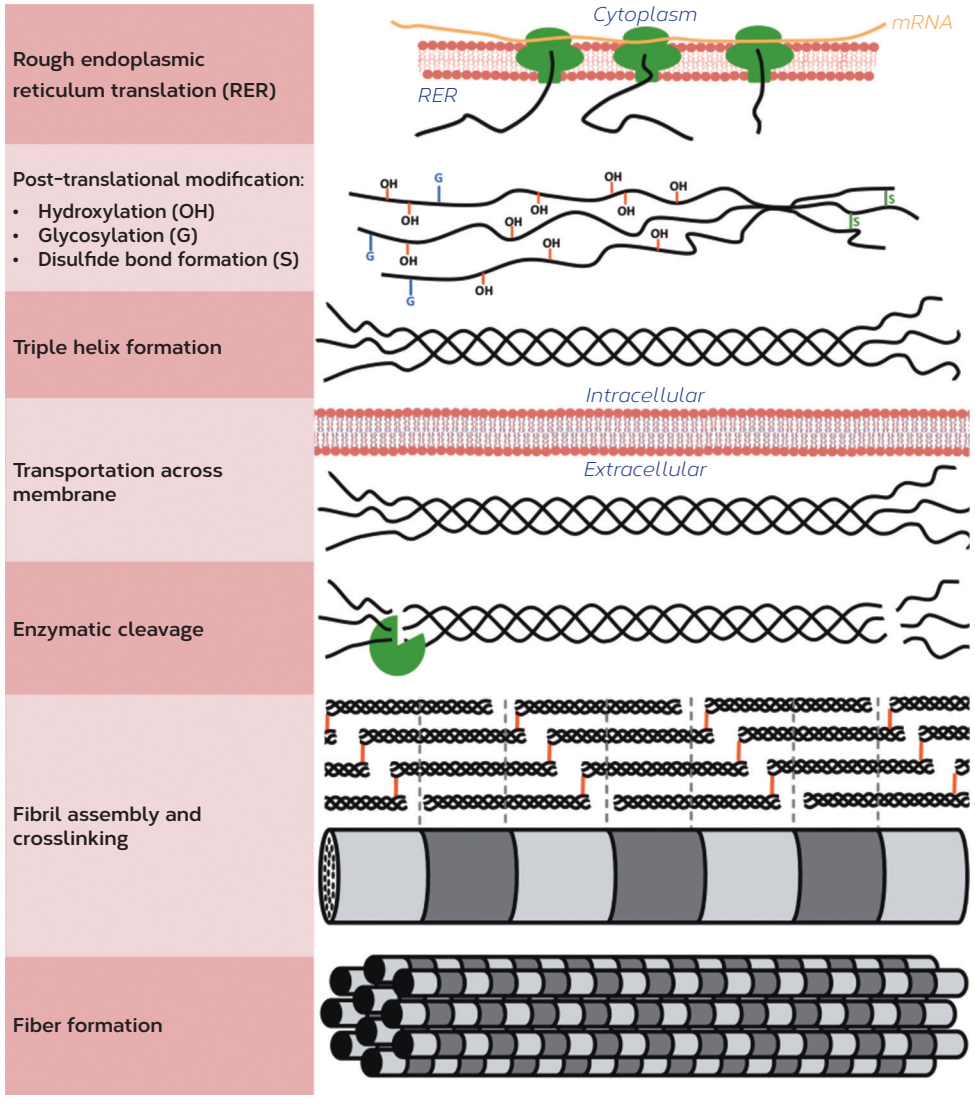

FIGURE 3: CARTOONIZED SCHEME OF THE SYNTHESIS AND STRUCTURE OF COLLAGEN.

As with all proteins, collagen mRNA is translated by ribosomes into individual polypeptides (pre-procollagen subunit) of which 3 assemble (procollagen) whilst undergoing posttranslational modification such as; hydroxylation, glycosylation and disulfide bond formation. The triple helix is formed and subsequently transported outside the cell where the N- and C-propeptides are cleaved off. The cleaved monomeric collagen (tropocollagen) is then involved in the self-assembly into collagen fibers with the characteristic quarter staggered array or banding pattern. The monomeric collagen overlaps approximately ¼ of each molecule, creating a dense and less-dense area, also referred to as the overlap D-periodicity. The monomers are subsequently fixed by crosslinks. In most cases the fibrils bundle and form larger collagen fibers.

65-70 nm (depending on the tissue) and is also referred to as the D-period.[48] The overlapping arrangement of the collagen molecules are stabilized by the formation of intermolecular covalent crosslinks. Most crosslinks are formed between N- and C-telopeptide ends and certain residues within the triple helical part of other neighboring molecules. During fibril formation non-covalent and reducible crosslinks are also formed between the molecules, based on either lysine aldehydes (aldimine bonds), hydroxylysine aldehydes (ketoimine bonds) or a combination thereof.[49] During maturation of the tissue, these crosslinks are converted into non-reducible bonds.[50] The N-telopeptide is believed to play an important role in regulation of fiber thickness, although this process is not yet fully understood.[51]

Other insights in collagen synthesis have led the community to believe that the incorporation of other types of collagen (i.e. type V, IX, XII or XIV) or other non-collagenous proteins during the fibril formation controls fibril thickness.[52] The fibril diameter and the distribution of different fibril dimensions is an important determinant of the mechanical properties of the tissue in question. Thick fibrils exhibit great tensile strength but are susceptible to creep (plastic-deformation). It has been suggested that thinner fibrils may be more efficient at inhibiting creep due to the larger surface area in contact with the rest of the matrix over which the shear stress dissipates.[53] Every tissue and inherent function has thus a specific distribution of collagen fibers with varying diameters.[54] In most tissues, collagen fibrils bundle to form even thicker collagen fibers.[55] These bundles of fibrils (fibers) are not connected to each other in a similar manner as the molecules within the fibrils. Instead the structure can be viewed as a highly interwoven set of fibrils/fibers.[56]

4.1.1.2 COLLAGEN APPLICATIONS

Collagen is regarded as one of the most versatile biomaterials in both hard and soft tissue engineering. Along with the mechanical and biological role in the ECM in both homeostasis and wound healing, the ubiquitous occurrence of collagen throughout the human body makes it an ideal candidate for use as a biomaterial. Due to its natural role in the ECM, type I collagen possesses worthy mechanical characteristics for a protein.[57] Fibrillar collagen is considered as a robust protein, which can endure relatively high temperatures and is, to a certain extent, resistant to corrosive

processing chemicals like salts, acids, bases and detergents.[58] Its general robustness makes collagen compatible with many processing techniques. The properties of collagen can be influenced using chemicals, enzymes and physical processes like drying or heating.[59] For example, mechanical and biochemical characteristics can be modified by crosslinking (see section 5.7) or functionalized by covalent addition of glycosaminoglycans.[60] One of collagen's unique properties is the reversible process of pH driven precipitation/fibrillation.[61] Collagen present at pH values where the net charge of the collagen molecules is either maximally negative or positive causes the molecules to repel each other and subsequently increase water uptake capacity. This phenomenon is referred to as 'swelling' and can be utilized in modification of its properties (figure 4).[62] Swollen collagen can be subjected to homogenization techniques to create a viscous fibrillar paste, which can then be formed into different scaffold types (see section 5 of this chapter).

Fibrillar collagen has been studied in numerous RM applications where biodegradability, porosity and mechanical strength were required.[36, 63, 64] Moreover, fibrillar collagen at an acidic pH can subsequently be digested to yield a truncated form of molecular collagen where the telo-peptide ends are cleaved using pepsin, resulting in atelocollagen.[65] This monomeric collagen resembles the native form of molecular collagen (acid soluble collagen) and both exhibit characteristics differing from fibrillar collagen. Molecular or monomeric collagen is completely soluble in water or acid and subsequently forms a viscous solution. Under physiological conditions (pH, solutes, temperature, etc.), fibrillogenesis can occur where de novo collagen fibrils are formed, in turn forming a hydrogel. Collagen hydrogels, or hydrogels in general, have a wide array of applications which are listed in section 4.2. Although successful in various applications, scaffolds based on monomeric collagen suffer from non-physiological small fibril dimension and poor mechanical strength.[66] Depending on the ultimate goal, the previously discussed items should be taken into consideration when working with collagen as a biomaterial.

4.1.2 GELATIN

Gelatin, a soluble protein derived from the partial unfolding or hydrolysis of collagen, is highly biocompatible and biodegradable in a physiological environment.[67] The

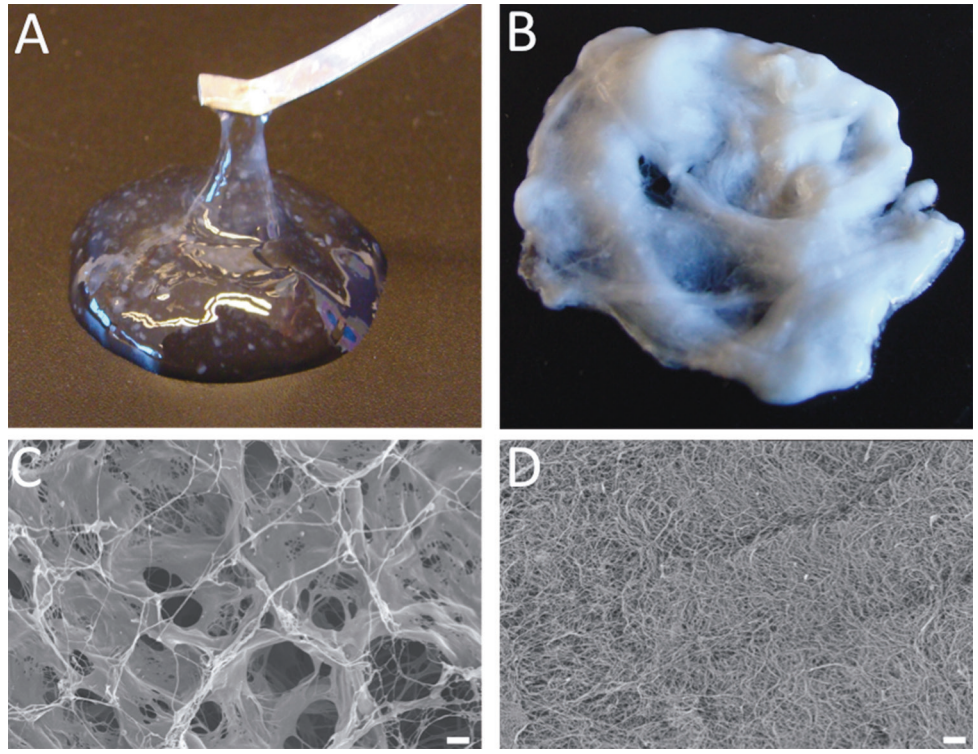

FIGURE 4: REVERSIBLE SWELLING AND PRECIPITATION OF COLLAGEN FIBERS.

A) 0.5% w/v swollen collagen fibril suspension in 0.25 M acetic acid at pH 3.0. **B)** Collagen that was precipitated by neutralizing the suspension to pH 7.4. SEM images show **C)** the acid swollen collagen and **D)** the precipitated collagen after freezing and freeze-drying displaying either a porous or fibrous structure, both bars represent 10 μm.

conversion of collagen into gelatin, or also referred to as a helix-to-coil transition, has been extensively studied in various settings.[68] Basically, gelatin is a mixture of collagen α-chains (single peptide chain), β-chains (two α-chains) and γ-chains (three α-chains) or any intermediates thereof. Depending on the state of the collagenous material, be it in fibers, fibrils or single molecules, a combination of acids, bases and heat treatment can be used to break the covalent and non-covalent bonds in order to destabilize the collagen triple helix.[69] The treatment method subsequently affects the final properties and generally results in either of two types of gelatin, commercially known as type-A gelatin (acid treated, isoelectric point between pH 8 and 9) and type-B gelatin (base treated, isoelectric point between pH 4 and 5).[70] Gelatin has a unique combination of properties for a protein, which make it a versatile biomaterial, i.e. from thermo-reversible gels to porous materials, films, capsules and electrospun fibers[71]. Gelatin is generally regarded as having low-antigenicity and the metabolic products thereof are harmless since collagen is normally degraded by proteolytic hydrolysis in the body.[72] Due to the aforementioned reasons, gelatin has been frequently used in drug carrier and delivery systems[73], as a plasma expender[74], wound dressing[75], medical device coating[76] and as a basis for RM applications in sponges, films and hydro/cryogels[77]. Gelatin has many applications within RM and is often combined with other carbohydrate-based biomaterials (e.g., chitosan, alginate and agarose) and an array of processing techniques (electrospinning, coacervation and crosslinking).[78]

4.1.3 ELASTIN

Elastin is a vital ECM protein that provides elasticity to tissues and organs.[79] Elastin comprises up to 70% of the dry weight in elastic ligaments, 50% in large arteries, 30% in lung and 2-4% in skin, where the fibers are present as rope-like structures.[80] Its primary role is to allow tissues to undergo repetitive deformation and subsequently facilitate the return to its original dimensions when the force is released.[81] It is generally accepted that the spontaneous recoil of stretched elastin is entropic in origin, where in its extended mode all the energy is taken up by the polypeptide backbone and is recovered upon relaxation.[82] Water-soluble tropoelastin monomers are alternatively spliced polypeptides with sizes ranging from 60-70 kDa. The monomers have characteristic alternating hydrophobic and crosslinking domains. When the tropoelastin molecules are transported outside the cell, the hydrophobic domains regulate the association with other tropoelastin molecules and the lysine and alanine-rich hydrophilic domains ensure the presence of free amine groups for subsequent crosslinking by lysyl oxidase to form insoluble elastin.[83] Unique to elastin is the formation of desmosine and isodesmosine, where four (modified) lysine residues from two tropoelastin molecules react with each other to form tetrafunctional crosslinks.[84] Elastin molecules are abundantly crosslinked to stabilize the structure and induce the elastic coil properties. Elastin has also been identified as a signaling molecule, governing cellular responses like chemotaxis, proliferation and differentiation.[85] Elastin can interact directly with cells through different cell-surface receptors like the elastin/laminin receptor which has been implicated in elastin assembly, cellular processes and interaction with other ECM components like GAGs, fibrillin and laminin.[86]

Due to its versatility, elastin has been subject of investigation for use in RM applications. However, due the extensive crosslinking and subsequent insolubility, elastin is very difficult to manipulate limiting its use as a biomaterial. Elastin can be solubilized by enzymatic or chemical hydrolysis to make it more suitable for use in scaffolding. However, along with the solubilization, the elastin partially loses its mechanical properties. The soluble elastin can still functionalize biomaterials. Soluble elastin can be coated on natural or synthetic polymers to modify the surface hydrophobicity[87], decrease thrombogenicity[88], increase angiogenesis[89] and improve the cell adhesion properties.[90] Three-dimensional scaffolds have been made from solubilized elastin as a hydrogel-based material[91] or as an electrospun sheet or tube.[92] Incorporation of both soluble and insoluble elastin in scaffolds has been investigated for use in dermal[93] and vascular applications.[94] For more elaborate description of the applications of elastin-derived biomaterials the readers are referred to excellent reviews by Almine *et al*.[95], Daamen *et al*.[96] and Sivaraman *et al*.[97]

4.1.4 ADHESIVE GLYCOPROTEINS

Cells adhere to the ECM through interaction between cell surface proteins and adhesive ECM glycoproteins, including fibronectins, laminins, fibrinogen, entactins, vitronectin, thrombospondins, tenascins, nephronectin, and others.[98] Most adhesive glycoproteins

play an important role in the cell attachment, movement and differentiation.[99] Cell surface proteins (mostly integrins) recognize certain domains within the adhesive glycoproteins for example the tripeptide sequence Arg-Gly-Asp (RGD). This sequence is crucial for the interaction of the adhesive glycoprotein with its respective cell surface receptor.[100] Next to cell surface proteins, adhesive glycoproteins possesses multiple binding domains capable of binding other ECM components like collagen, elastin, proteoglycans, GAGs and other adhesive glycoproteins.[101] This section will shortly describe the adhesive glycoproteins laminin, fibronectin and fibrinogen, which are of interest to RM applications. Other adhesive glycoproteins have received much less attention as of yet due to their relatively unknown role in organogenesis. These include vitronectins, thrombospondins, tenascins, entactins and nephronectin, which all have their specific effects on cell and protein binding, ECM remodeling and wound healing.[102] Moreover, glycoproteins can be involved in tissue transplant rejection and this should be taken into consideration in designing RM applications.[103] Taking specific peptide sequences responsible for a certain functions of the respective proteins, e.g., adhesion, migration or proliferation, can circumvent immunogenic responses against these adhesive glycoproteins.[104, 105] Next to the previously mentioned RGD, the YIGSR and IKVAV sequences are also used for cellular adhesion to surfaces.[106, 107] Also, short sequences from collagen[108] and proteoglycans[109] are being used in similar manners to controllably elicit desired cellular responses. Straightforward peptide chemistry could be used to synthesize functional short polypeptides. Hence, expensive preparation methods of full lengths proteins such as purification from an animal sources or recombinant DNA techniques could be circumvented.

4.1.4.1 LAMININ

Laminins are considered to be one of the major adhesive glycoproteins and are a main component of the basement membrane. Laminins are large heterotrimeric glycoproteins consisting of α, β and γ chains. Due to alternative mRNA splicing different chains can be synthesized respectively. Around fourteen chain combinations have been described *in vivo* and each molecule is named according to their chain composition.[110] It is generally believed that the cross-like structure of laminin facilitates both cell adhesion as well as laminin sheet or basement membrane formation. Additionally, laminin has a high affinity for type IV collagen and heparan sulfate, which are also major components of the basement membrane.[111] Laminins generally play important roles in cell adhesion, migration, proliferation, neurite outgrowth, and angiogenesis.[112] Due to its high affinity for ECM components and cell surface proteins, laminin is widely used in the coating of cell culture surfaces or scaffolds.[113]

4.1.4.2 FIBRONECTIN

Fibronectin shows functional similarities with laminin in its roles in the ECM. Fibronectin is a protein dimer produced from a single gene and consisting of nearly identical monomers that are linked by disulfide bridges. Each subunit contains three types of repeating modules (FN1, FN2 and FN3) linked by short connector modules. Due to alternative splicing, which is regulated by cell type and stage of development, differences in these subunits are induced and have resulted in over 20 types of human fibronectin variants.[114] Early on in ECM formation, fibronectin is assembled into a fibrillar network on the cell surface providing deposition points for fibronectin-interacting ECM proteins like collagens, fibrin and heparan sulfate proteoglycans.[115]

4.1.4.3 FIBRINOGEN

The last major adhesive glycoprotein is fibrinogen. Fibrinogen is a large (349kDa), soluble and complex protein consisting of three different subunits (Aα, Bβ and γ) varying in amino acid length (610, 461 and 411, respectively) linked by disulfide bonds.[116] Fibrino-peptides on the soluble fibrinogen can be cleaved by thrombin to convert fibrinogen into insoluble fibrin. Fibrin molecules assemble to form a protofibril, which subsequently aggregate into larger fibers. A 3D network, or a fibrin clot, is formed which is capable of catching blood platelets to promote aggregation. This is essential for forming a hemostatic barrier and the newly formed network offers temporary scaffolding for cells involved in wound healing. Transglutaminase (factor XIIIA) crosslinks the glutamine and the lysine residues in the fibrin clot which temporarily stabilizes the network against chemical, mechanical and proteolytic degradation.[117] Fibrin, or forming fibrin clots, also has affinity for some plasma proteins such as fibronectin and albumin, which have influence on properties like

fibril thickness and density.[118] Other (glyco)proteins like thrombospondin, von Willebrand factor and fibulin bound to the fibrin clot increase the affinity for blood platelets. Moreover, growth factors like FGF-2 and VEGF are also bound to stimulate swift remodeling of the damaged site. It is noteworthy, that because of the presence of other proteins, clots formed *in vivo* have different properties compared with clots formed with purified components.

4.1.4.4 APPLICATION OF ADHESIVE GLYCOPROTEINS

Laminin, fibronectin and fibrinogen have received much interest from the RM community due to their innate role in wound healing and ECM formation.[119] Also their role in cell-cell and cell-ECM adhesion during both tissue growth and tissue homeostasis makes them useful components in RM. Being a minor ECM component and potentially immunogenic, laminin is less suitable for use as a structural scaffolding but more so as a coating material. The most frequent use of laminin can be traced to *in vitro* culture of various cell lines and primary cells.[120] In TE of the central nervous system, both synthetic[121] and natural materials[122] have been coated with laminin to improve cellular compatibility.[123] Laminin has also been incorporated in hydrogels to improve cell adhesion in central nervous system[124], intervertebral disc[125] and pancreas[126] applications. As fibronectin also increases cellular adhesion, it has been used in similar applications as laminin.[127] Some effort has gone out to link fibronectin to biomaterials via laser patterning[128], chemical conjugation[129] and genipin crosslinking[130]. Fibronectin has also been used frequently in combination with fibrinogen as a scaffolding material.[131] Perhaps the most versatile of the adhesive glycoproteins is fibrinogen and/or fibrin. Due to its innate clotting ability after cleavage by thrombin, it is highly suitable for use as a surgical adjuvant in hemostatic, sealing or adhesive applications.[132] The use of fibrin in modern medicine has therefore received most attention and has been applied in the clinic extensively.[133] The versatility of fibrinogen/fibrin has been demonstrated by its compatibility of a wide range of scaffolding techniques including, but not limited to, hydrogel formation[134], electrospinning[135], coating[136], particulate leaching[137] and others. The use of fibrin has also been researched for nearly every TE application including adipose[138], bone[139], cardiac[140], cartilage[141], muscle[142], neural[143], ocular[144], respiratory[145], skin[146], tendon[147], ligament[148] and vascular tissue.[149] For further reading, several excellent reviews of fibrin in TE are available.[150,151]

4.1.5 KERATIN

There are countless other proteins inside and outside the cell that were not discussed in the previous text. As proteins can be classified by their shape as for example fibrous or globular, the structural proteins are usually regarded as fibrous.[152] Despite being the main component of horse tail-hair, an early naturally occurring suturing material, the fibrous protein keratin, has enjoyed only marginal research for RM applications.[153, 154] Keratins are epithelial-specific family members of the superfamily of intermediate filament structural proteins that are of paramount importance in the outer layer of the skin, hair, nails and horns. The keratin sub-family comprises of types I and II, of which respectively, 28 and 26 genetic variations are known. Keratins fulfill two main fundamental roles in epithelial cells where they provide structural support and participate in the regulation of metabolic processes like proliferation, migration and apoptosis.[155] The soluble keratin monomers assemble into bundles and in turn form insoluble intermediate filaments.[156] The exact process of assembly remains unclear, but for further information the readers are referred to an excellent review by Kölsch *et al*.[157]

Keratin has been considered as a biomaterial by several research groups due to several key features. For example, structures containing large amounts of the assembled keratin filaments are generally thermostable and possess great mechanical strength.[158] Evidence also suggest that keratin is biocompatible and does not induce obvious toxic effects both *in vitro* and *in vivo*.[159] Moreover, keratin-based biomaterials have an advantage in that they do not degrade by the same mechanisms as other proteins because of the lack of keratinases in mammals. The breakdown of keratin-based biomaterials is regulated by phagocytosis and subsequent ubiquitin systems.[160] This subsequently allows keratin-based scaffolds to persist longer than other protein-based scaffolds.[161] Keratin-based biomaterials in the form of sponges and films have been produced from wool and human hair for various biomedical applications such as wound dressings a neural tissue engineering applications.[162, 163] Keratin can also be processed into a hydrogel and has shown to support nerve cell migration and

proliferation.[164] Keratin-based hydrogels in combination with commercially available nerve conduits have been used in small mammal studies to replace peripheral nerve segments and proved to be equivalent or more effective autografts.[161, 165] The use of keratin for its great mechanical strength has also attracted interest from the hard tissue arena, where it has also shown to a versatile biomaterial.[166, 167]

4.1.6 PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

Proteoglycans (PGs) are a class of proteins that harbor one to many glycosaminoglycans (GAGs). A proteoglycan consists of a core protein to which GAGs are covalently attached. PGs influence and help regulate ECM assembly and collagen fibril formation.[168] This is important during inflammation, tissue repair and remodeling. Moreover, PGs play an important role in water homeostasis of a variety of tissues and contribute to the mechanical resilience in joints. A number of other functions include regulation of coagulation, lipoprotein clearance in the liver, control of growth factor binding, and signaling. As in adhesive glycoproteins, PGs participate in both cell-cell and cell-matrix interactions. The attached GAGs largely determine the specific properties of a PG. GAGs are long, linear and strongly negatively charged polysaccharides, characterized by up to quantity hundred or so repeating disaccharides. GAGs can be either sulfated or non-sulfated which also largely determines the overall PG charge.

Sulfated GAGs are usually classified into five types: heparan sulfate (HS)[169], heparin[170], chondroitin sulfate (CS)171, dermatan sulfate (DS)[171] and keratan sulfate (KS)[172]. Hyaluronan (HA) is the only non-sulfated GAG[173]. Important PGs include perlecan (basement membrane, HS and CS)[174]; aggrecan (cartilage, CS)[175]; versican (connective tissue, CS and DS)[176]; neurocan and brevican (CNS tissue, CS)[177, 178]; biglycan, fibromodulin and decorin (collagen rich tissues, CS and DS)[179, 180]; bikunin (plasma, CS)[181]; lumican, keratocan, glypicans and syndecans (epithelial tissues and cornea, HS and KS)[182, 183]. The structural diversity of the PGs and its respective GAG composition renders each PG and GAG unique in its biological function. Because of the ubiquitous presence of GAGs in healthy tissue ECM and their diverse functions in tissue homeostasis, GAGs have gained interests from the biomaterial researchers. In RM, GAGs are frequently used to supplement existing scaffolds, made from natural materials, synthetic polymers or a combination thereof.[184]

4.1.6.1 HEPARIN AND HEPARAN SULFATE

Heparin and heparan sulfate (HS) are highly sulfated GAGs where heparin is associated with the highest negative charge density of any known biological molecule.[185] Native heparin and HS are, like other GAGs, polymers with varying molecular weight (10-70 kDa and 7-20 kDa, respectively).[186] They consist of disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid, of which the latter sugar can be epimerized into L-iduronic acid during subsequent variable modification of the polymer. Other modifications include N-deacetylation/N-sulfation, 6-O and 3-O sulfation of the glucosamine and 2-O sulfation of the uronic acid. Alternatively, the glucosamine can have a free amine group on the C2position instead, likely due to uncoupling of the N-deacetylation and N-sulfation.[187] Together with 3-O sulfation, this is the least prevalent modification. Heparin and HS are closely related due to the composition of the disaccharide structure, however the main difference being that heparin has about a two-fold increase in N- and O-sulfation and subsequently two times its overall negative charge.[188] Whereas heparin is almost uniformly highly sulfated, HS contains domains of low, moderate and high sulfation.[189] The exact function of heparin and HS in the human body is still being elucidated but current literature holds plenty of evidence suggesting that they are both involved in a wide array of functions. Heparin is mainly produced and stored by basophils and mast cells, whereas HS can mainly be found on the cell surface and in the surrounding ECM. Heparin is used as a pharmaceutical anticoagulant. Medical devices that come into direct contact with the blood circulation are often coated with heparin to prevent blood clotting. Within the RM theme, heparin is utilized for its ability to bind an array of growth factors. Growth factors with heparin binding domains can be attached to heparin immobilized on a scaffold surface.[190] The attachment of heparin and subsequently heparin-binding growth factors like FGF2 and VEGF has shown to increase the angiogenesis in subcutaneously implanted collagen scaffolds.[60] Additionally, the attachment of heparin to (non-)biodegradable materials can influence the tissue response where the negative charge can influence the water holding capacity or the overall hydrophobicity of a construct. For further reading regarding the exact differences between heparin and HS the readers are referred to other sources.[191,192]

4.1.6.2 CHONDROITIN SULFATE

Chondroitin sulfate consists of two alternating monosaccharides, D-glucuronic acid and sulfated N-acetyl-D-galactosamine. With a molecular weight ranging from 20-60 kDa, CS is the most prevalent GAG in the human body and is an important component of cartilage where it plays a role in resistance of compressing forces.[193] CS comprises a group of 8 CS subtypes, CS-A, C, D, E, F, H, K, and O, of which the type is dependent on the sulfation pattern of the monosaccharides. The sulfations at different positions of the monosaccharide confer different biological activity to the CS-containing PG.[194] For example, in cartilage CS containing PGs like aggrecan are tightly packed with the collagen structure where the charged sulfate groups of the CS generate electrostatic repulsion that provides resistance to compression. For further details regarding the different subtypes the reader is referred to an excellent overview.[195] The main medical application of CS lies in nutraceutical application for the treatment of osteoarthritis. Along with glucosamine, CS has been thought to pose anti-inflammatory properties although the possible underlying mechanism is poorly understood.[196] Within RM, CS has been subject of research for use in scaffolds to improve the biomechanical properties.[197] However, CS is currently mostly limited to osteochondral and cartilage applications.[198] Like heparin (and HS), CS is able to bind growth factors and is therefore also able to modulate the bioactivity of the material.[199] CS has also found its way into a commercially available biomaterial application for skin regeneration by Integra Life Sciences, where a bilayer wound matrix consisting of type I bovine collagen bound CS and silicone is applied as a wound dressing.[200]

4.1.6.3 DERMATAN SULFATE

Dermatan sulfate is composed of disaccharide units consisting of N-acetyl-D-galactosamine and glucuronic/iduronic acid. Like CS, DS is defined by the presence of N-acetyl-D-galactosamine, however, the presence of iduronic acid distinguishes it from CS. DS is the predominant GAG expressed in the skin but is also found in blood vessels, heart valves, tendons and lungs. Several processes involve DS as a co-factor or regulator of growth factors, cytokines, and chemokines.[201] Moreover, DS plays a n important role in signaling in response to the coagulation cascade and cellular damage

and subsequent regeneration of the fibrous ECM.[202] DS as a biomaterial or component of biomaterials have enjoyed little attention.[35] Several attempts have been made to study the *in vitro* effect of DS on chondrocyte, fibroblast and keratinocyte function.[203] It is possible that difficulties in procurement of large quantities have hampered widespread study of DS in TE/RM applications. For further reading regarding the function of DS, an extensive overview is given by Trowbridge *et al*.[204]

4.1.6.4 KERATAN SULFATE

Keratan sulfate (KS) is relatively the simplest of the sulfated GAGs, consisting of repeating disaccharides composed of galactose and N-acetyl-D-glucosamine with sulfation at the 6-O positions. The size of KS is highly variable and can range from 2 to 24 kDa.[205] KS is the major GAG in the cornea and can also be found in scar tissue of the central nervous system and in connective tissues like cartilage.[206, 207] During the formation of the cornea, KS interacts with collagen to assure the characteristic highly organized collagen fibril structure, which renders it transparent.[208] Like other GAGs, KS has water-binding capabilities and thus plays an important role in the maintenance of the transparency and general homeostasis of the cornea. The water binding in combination with fibrous proteins enables the tissue to endure mechanical wear and tear from compression and abrasion. KS has also been linked to the repair of corneal tissue where it is believed to paradoxically act as a cell de-adhesion molecule but also to facilitate the motility and attachment of the corneal epi- and endothelial cells.[209, 210] The application of KS in RM research has to date been limited to use as coatings in cell culture to investigate cell motility.[210] However, synthetic analogues to proteoglycans (peptidoglycans) have been used more frequently in scaffold design.[211] For further reading please see some excellent literature on KS and the physical and biological aspects.[212, 213]

4.1.6.5 HYALURONIC ACID

Hyaluronic acid, also referred to as hyaluronan or hyaluronate, is an anionic non-sulfated GAG and is composed of repeating disaccharide units containing D-glucuronic acid and N-acetyl-D-glucosamine. HA is a major constituent of synovial fluid but is also

found in connective, neural and epithelial tissues. HA is unique from other GAGs since it is non-sulfated, not attached to a core protein and has a greatly varying molecular weight which can range from 1 kDa to 10,000 kDa.[214] Like other GAGs, due to its negative charge, HA is able to bind large amounts of water which allow it to function as a space filler and biological lubricant of joints.[215] HA based applications have been used since the 1980s as a surgical aid for eye-related surgery. Since then it has been used in viscosupplementation treatments, where HA is injected to supplement synovial fluid in (osteo)arthritic joints.[216] Early in the 2000s, HA became a popular ingredient for cosmetic surgical applications like injectable fillers for facial wrinkles. In the last decade, HA has enjoyed gaining popularity as a biomaterial due to its biocompatibility and biodegradability in combination with its gel-forming capabilities. HA can be crosslinked directly using formaldehyde or divinyl sulfone to form a weak but stable hydrogel. However, HA can also be modified by attaching thiols, methacrylates and tyramines to create self-gelling systems.[217, 218] HA is also a component frequently used in combination with fibrous proteins like fibronectin and collagen.[219]

4.2 NON-MAMMALIAN-BASED ECM MATERIALS

The biomaterial armamentarium is filled with many non-mammalian raw materials isolated from sources such as insects, (shell)fish, plants and microorganisms. In this section a selection of frequently used biomaterials is given, most of which are based on polysaccharides.[220] Countless mammalian proteins that have been successfully produced in transgenic expression systems, using both mammalian and non-mammalian hosts, are omitted in this review.

4.2.1 SILK FIBROIN

Silk is a naturally occurring protein polymer produced by a wide variety of insects and arachnids.[221] The natural function of silk is generally to make webs, cocoons and draglines. In its natural form, silk is composed of a filament core protein, silk fibroin, and can be coated with glue-like sericin proteins.[222] The composition of different silk types vary depending on the organism and subsequent function.[223] However, all silks have a similar structure composed of hydrophobic and hydrophilic blocks.

The hydrophobic blocks are highly conserved and have a repetitive sequence of short-side chain amino acids. The hydrophilic blocks form β-sheet structures and have more complex sequences that consist of larger side-chain and charged amino acids. The combination of the hydrophobic and hydrophilic blocks determines the mechanical properties.[224] It is due to the exceptional mechanical properties, that researchers in the field of biomaterials have gained great interests in harnessing its capabilities.[225] Additionally, depending on the exact species from which it is harvested, silk is thermostable up to 250°C which is extremely high for a protein structure.[226] Isolated silk fibroin is soluble in aqueous conditions which makes it compatible with many processing techniques.[227] The techniques used in combination with silk fibroin, ranges from simple casting and drying methods[228] to hydrogel formation[229] and from electrospinning[230] or fiber deposition[231] to microporous scaffolds made by salt leaching, gas foaming, freeze drying or freeze-thawing.[232-234] For further reading, please see elaborate reviews by Wang *et al*., Altman *et al*., Harkin *et al*. and Vepari *et al*.[225-227, 235]

4.2.2 ALGINATE

Alginates (also referred to as algin or alginic acid) comprise a broad family of polydisperse linear anionic polysaccharides naturally found in brown seaweeds and consist of varying sequences of β-D-mannuronic acid (M-blocks), α-L-guluronic acid (G-blocks) or mixed sequences (MG-blocks).[236] Typical alginate size ranges from 500-5.000 residues per chain (100 – 1000 kDa).[237] Alginate can chelate with divalent cations which results in ionic bond driven gelation, also referred to as the "egg-box-model".[238] The interaction is mainly driven by interaction of the G-blocks that form stronger bonds with the divalent cations than the M-blocks. Depending on the seaweed source and growing conditions, the ratio of mannuronic and guluronic acid can vary, thereby affecting the alginate biomaterial properties.[239] At this moment the exact sequence of alginate, if any, is unknown, and the differences can only be expressed as average values of amounts of the different M/G-block combinations.

Mainly due to the simplicity of the ion driven gel-forming capacity under physiological conditions in combination with its low cytotoxicity[240], the biomaterial community has taken great interest in alginate. Alginate is versatile and can be

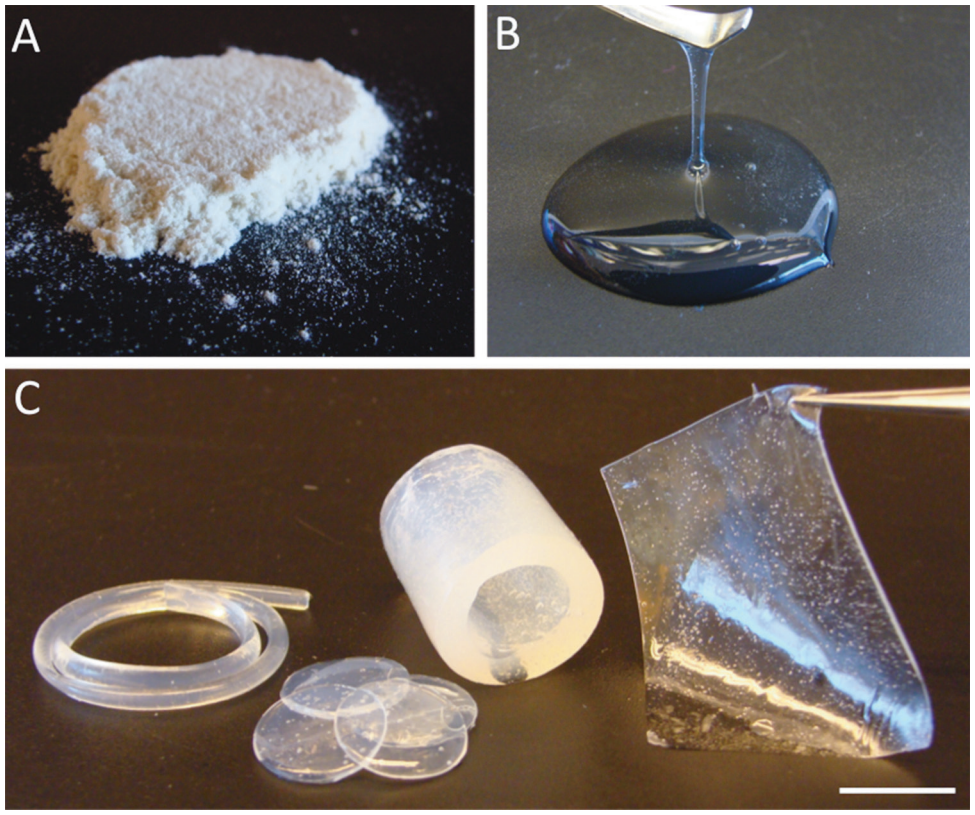

FIGURE 5: ALGINATE BIOMATERIALS.

A) Commercially available alginate powder. **B)** A 5% (w/v) alginate in phosphate buffer results in a viscous gel-like solution. **C)** Various scaffold shapes can be prepared by exposing the alginate solution to divalent cations, ranging from tubes and gels to films and massive strands bar represents 1 cm.

subjected to other processing methods including, but not limited to, freeze-drying[241], electrospinning[242], 3D printing[243], and other chemical modifications or crosslinking methods like, phase transition[244], cell crosslinking[245], click reactions[246], and free radical photo-polymerization[247]. Moreover, due to its gelling properties, alginates are highly useful in the design of microspheres for delivery of cells, growth factors, genes and other drugs.[248, 249] A vast array of alginate-based scaffolds and other devices have thus been made, however most cells require peptide sequences to attach to certain surfaces (figure 5). Since alginate is a polysaccharide it may require modification for certain cells to be able to adhere.[250] This can be achieved by attaching RGD or other peptides sequences to the alginate chains using carbodiimide crosslinking.[251] This technique couples the terminal free amine group of a protein sequence to the carboxylic group in alginate.[252] To further enhance the biocompatibility, alginate has been extensively used in combination with other biomaterials like decellularized ECM powder[253], GAGs[254], collagen[255] and other carbohydrates.[256] For further details regarding the extensive subject of alginates the readers are referred to several reviews.[257-259]

4.2.3 CHITOSAN

Chitin can be found in natural sources ranging from invertebrates, fungi, algae and yeasts.[260] Chitosan is a linear polysaccharide derived from the partial deacetylation of chitin and is composed of N-acetyl D-glucosamine and D-glucosamine units.[261] The deacetylation of chitin can vary from 40% to 100% and the molecular weight depends on the source and preparation method (300 to 1000 kDa).[262] The deacetylation is generally achieved by the use of chemical (alkaline) or enzymatic hydrolysis.[263] The chitosan properties can be affected by the degree of (de)acetylation which decreases the acetyl groups and increases the number of free amine groups on the C2 or C5 position of the D-glucosamine molecules. 40% of the acetyl groups in chitin should be removed before it can be referred to as chitosan.[264] The resulting free amine groups in the chitosan structure can be protonated, making it more soluble than chitin at slightly acidic conditions.[265] The free amine groups makes chitosan a positively charged polysaccharide.[266] Additionally, since amine groups are often involved in ionic bonds and formation of covalent peptide bonds, it makes chitosan suitable for hybrid products with other protein-based and synthetic biomaterials.[267]

Numerous studies focusing on chitosan and chitosan hybrid materials have indicated low cytotoxicity, biocompatibility and biodegradability.[268] These properties have attracted attention from the biomaterial community and have led to study of various applications in for example drug delivery[269], gene therapy[270], RM[271] and hemostatic applications[272]. For more detailed overviews please see these reviews.[261, 273, 274]

4.2.4 OTHER POLYSACCHARIDES

Next to the larger class of biomaterials being used for RM purposes, like GAGs, alginate and chitosan a group of polysaccharides such as dextran and cellulose, remain underutilized. The biological activity and biocompatibility of polysaccharides have been demonstrated in other applications dealing with various cell culture applications. However, polysaccharides are attracting attention due to their capability to form hydrogels. Depending on the polysaccharide the hydrogel characteristics can vary and have uses in different areas. Glucose-based polysaccharide biomaterials include starch, dextran and cellulose, where the differences are noticed in the way the glucose molecules are bound or if they are linear or branched. Dextran has long been used as an antithrombotic agent by reducing the viscosity of blood. However dextran has also been mixed with other biomaterials to modify the gelation properties for scaffolds used in an array of applications.[275-277] Starch has also found its way as an ingredient of biomaterials that modify the stiffness of the scaffold in question.[278, 279] Cellulose is naturally a very strong and versatile polysaccharide and, due to its crystalline structure, has been mainly used for hard tissue engineering.[280] More complex polysaccharides like agarose and pectin consist of less ubiquitous saccharides like D-galactose and derivatives thereof in the case of agarose. Pectins consist of mainly D-galacturonic acid with appendant residues such as D-xylose, D-adipose and D-arabinose. Both agarose and pectin are slowly starting to find its way into biomaterials for RM.[104, 281, 282]

5. TECHNIQUES AND MAJOR TOOLS FOR SCAFFOLDING

Running parallel to the emergence of new biomaterial ingredients is the development of techniques to produce scaffolds or tissue engineered constructs from these very same ingredients. In comparison to decellularized materials, molecularly defined materials can be transformed into a wide array of shapes and sizes, often with a controlled morphology. This is also the main advantage in using molecularly defined ingredients, where the morphology can be adjusted to the application in question. Moreover, in contrast to decellularized materials, the increase in standardization and reproducibility should eventually lower the cost of production and thus the eventual treatment modality. In general, molecularly defined scaffolds possess morphological characteristics having a porous, hydrogel or fabric-like structure.[283] All types of scaffolds have their own advantages and limitations.[20] Ideally, the mechanical properties of the biomaterial in question should match those of the target tissue but should simultaneously passively or actively stimulate cell growth, migration and differentiation. Often an optimum between porosity and the correct mechanical properties is sought after. The porosity is important to the functioning of the scaffold because it impacts the ability of the cells to migrate and nutrients to diffuse throughout the scaffold. In this section different tools are described which can be used to create scaffolds from natural ECM-based sources with control over the morphology.

5.1 POROUS MATERIALS

Porous materials represent one of the largest classes of molecularly defined scaffolds currently being researched. Methods and fabrication technologies to produce porous scaffolds are widely available for both natural and synthetic polymers.[284,285] Next to the obvious requirement that the scaffold should support cell growth, the idea behind the use of porous scaffolds consists of two main reasons where the pores should facilitate cell infiltration and the supply of nutrients. By inducing pore-like structures,

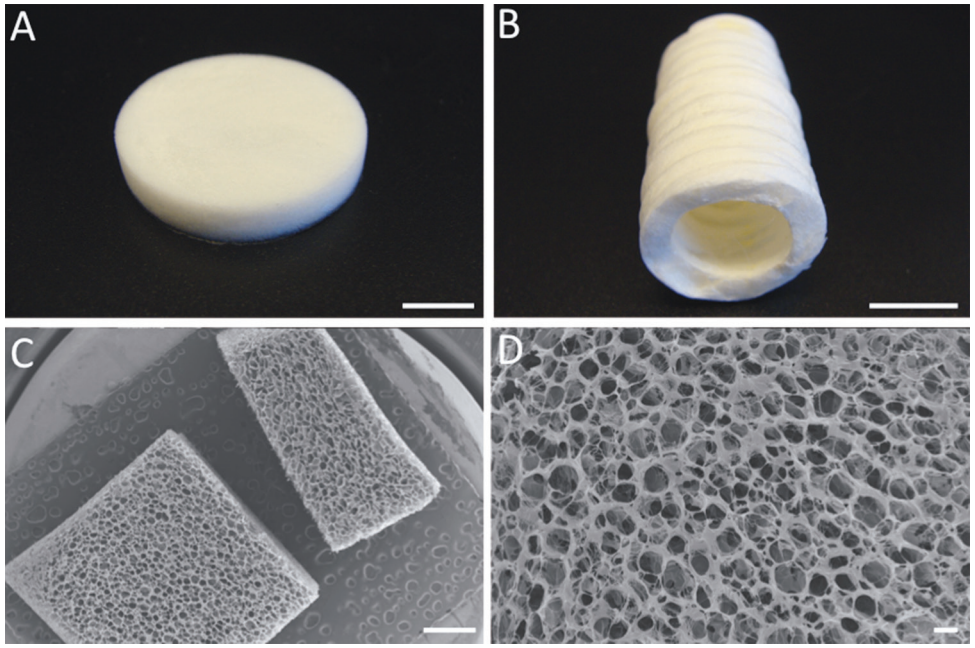

FIGURE 6: POROUS SCAFFOLDS.

A) Basic collagen scaffold made using a standard 6-well plate, freezing and subsequent freeze-drying, bar represents 1 cm. **B)** Collagen scaffold made in a similar fashion but with a different mold consisting of a tube as outer container and a mandrel to create the lumen, bar represents 1 cm. **C)** and **D)** SEM images of a basic porous collagen scaffold made using freezing and freeze-drying, bars represent 1 mm and 100 μm in C and D, respectively.

the surface area of the scaffold is greatly increased and should subsequently allow for cells to create their own microenvironment. An important requirement inherent to porous scaffold is the interconnectivity of the pores, which absence would hamper cell infiltration and nutrients diffusion.[286] Next to the porosity and interconnectivity, the shape and size of pores are important to accurately mimic the target tissue. In the body, ECM structures often have special arrangements or alignments to cope with a certain function. Depending on the application, porous scaffolds have been used for countless *in vitro* and *in vivo* RM studies. In the case of cartilage tissue, spatial differences in collagen fiber orientations provide the tissue with its properties: in the superficial zone collagen fibers are arranged parallel to the articular surface to optimally resist shear stresses during joint loading. In contrast, the orientation of collagen fibers in the deep zone is perpendicular to the articular surface, providing compressive strength to the tissue.[287] To impart porosity on biomaterials, e.g., porogens in combination with solvents[288], and phase separation in combination with solvent evaporation (precipitation[289], gas foaming[290], salt leaching[291], freezing and lyophilization[292]) are used. Novel techniques are emerging to make porous scaffolds, like stereo- and photolithography.[293, 294] An advantage of these processes is that they are promiscuous techniques, compatible with different materials and is easily adapted for straightforward scaling-up and subsequent lowered cost of production.

Using collagen as an example, different porous scaffold types can be made using homogenization and subsequent freezing processes (figure 6).[295] The shape into which the collagen gel was cast prior to the freezing process mainly determines the final macroscopical morphology of a collagen-based porous scaffold. The ultrastructure of a porous scaffold can be created with freezing and freeze-drying using most ECM-based materials, which are either soluble or easily dispersible. Materials less compatible with this method may require other methods to impart porosity as previously mentioned. The freezing temperature influences the formation of ice crystals (which become pores after lyophilization), including their size and orientation (figure 7).[296] In several tissues, the ECM is oriented either unidirectionally (e.g. cartilage) or radially (e.g. muscle fibers in the diaphragm). A scaffold with unidirectional pores can be created by applying a vertical temperature gradient to a collagen suspension (figure 7), which mimics the ECM of healthy cartilage.[295] A similar principle can be applied to guide the generation of muscle cells in the correct orientation using a scaffold with a radial pore

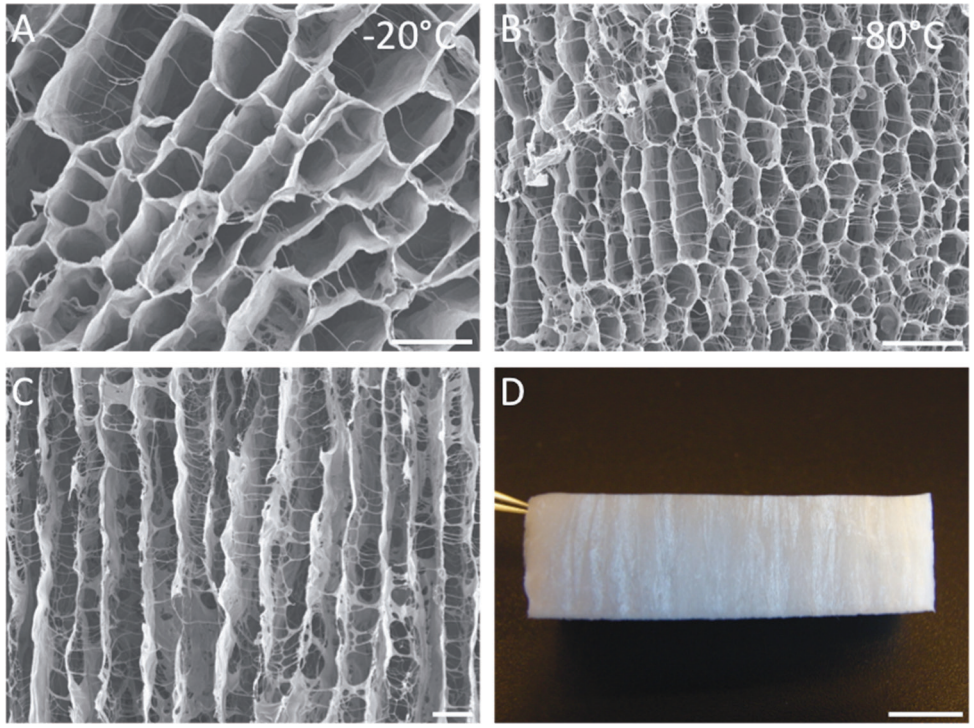

FIGURE 7: CONTROLLING PORE STRUCTURE.

A) Influence of temperature on pore size of a collagen scaffold frozen at -20°C and at **B)** -80°C, both bars are 100 µm. **C)** Ultra structure of a **D)** unidirectional collagen scaffold where the direction of the pores was controlled using directional freezing, pores are oriented from bottom to top. Bars represent 100 µm and 1 cm, respectively.

structure. This can be achieved by applying directional freezing from inside-out using a centrally positioned cooled tube.[297]

5.2 HYDROGELS

Hydrogels are defined as insoluble, three dimensionally (crosslinked) polymer network structures composed of hydrophilic homo- or hetero-co-polymers, which have the ability to absorb significant amounts of water.[298,299] As in porous biomaterials, hydrogels can be made from different natural and synthetic homo- and copolymers in combination with a variety of preparation methods. A key advantage of hydrogels is the preparation versatility, wherein the suspension can pre-seeded with cells before the hydrogel is formed, which results in homogeneous distribution.[300] The gels can be made using many materials e.g. collagen molecules[301], gelatin[302], elastin[303], alginate[304], silk-fibroin[305], cellulose[306], chitosan[307], hyaluronic acid[308] and others.[77, 309] However, hydrogels are generally soft structures lacking the mechanical properties necessary for many load bearing TE applications.[310] Hydrogels are mainly classified according to their physical structure; amorphous, semi-crystalline or hydrocolloidal.[311] The formation of the hydrogels is based on different techniques; covalent bonds produced by the reaction of one or more co-monomers, freeze-thaw cycles, physical crosslinks due to chain entanglement or enzymatic reaction, association bonds including hydrogen bonds or strong van der Waals interactions between chains, crystallites bringing together two or more macromolecular chains and de novo fibril auto assembly.[312, 313] Adjusting parameters like concentration, temperature or the application of shear forces or magnetic fields during the polymerization process (hydrogel formation) can influence the structure.[301, 314] For further reading the following reviews are recommended.[315-317]

5.3 FILMS AND COATINGS

Natural ECM components can be processed into thin films or coatings for other (bio) materials. Several applications might require scaffolds, which are non-porous or, even impenetrable for liquids and/or gases. Films are generally regarded as thin, non- or low-porous scaffolds with interesting mechanical properties.[266] Coatings can

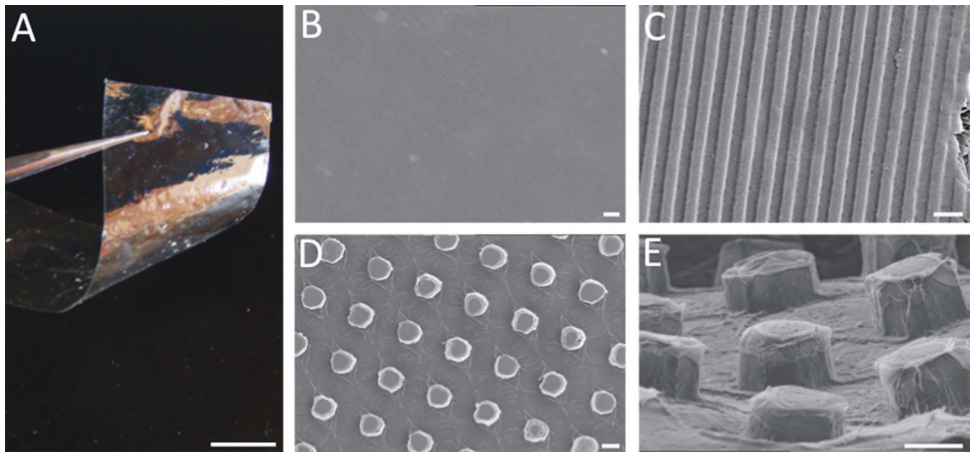

FIGURE 8: FILMS AND TOPOGRAPHY.

A) Macroscopical image of a collagen film made by air-drying, bar represents 1 cm. **B)** SEM image of a collagen film, bar represents 1 µm. **C)** Surface topography induced by varying casting shape, bar represents 1 µm. **D)** and **E)** Another possible variation of surface topography, made to mimic the rete-ridge structure between the dermis and the epidermis, both bars represent 100 µm.[330]

often have the same composition as films, but are mainly intended to add certain properties, like hydrophilicity and biocompatibility, to other (bio)materials.[318] The film components can be varied and used in different combinations to adjust its properties like transparency, strength, elasticity, nano-porosity, biodegradability and biocompatibility.[319, 320] The science behind design and production of films from natural ECM components is often based on the knowledge gathered from synthetic or natural polymeric films.[321,322] Synthetic film formation is frequently based on melting/dissolving and subsequent solidification of the polymer. However, the use of polysaccharides and relatively fragile proteins call for different approaches that avoid high temperatures and corrosive conditions. Soluble proteins and polysaccharides are often compatible with an array of techniques such as dip coating, spray coating, spin coating, or solvent casting and subsequent drying to produce films or to coat existing (bio)materials. Films have been made from many different raw materials including, but not limited to collagen[323], gelatin[324], keratin[163], silk[226], alginate[325] and chitosan[266]. Due to their high transparency, films have frequently been used in TE of the cornea[326], but also for nerve regeneration[327], engineering of skin and adipose tissue[328,329]. In some applications the films require certain structure or topography to stimulate directional growth and differentiation of the cells (figure 8). Techniques have been developed which can induce structure using micro-molding[322], casting[330], and laser patterning[331]. For further details the readers are referred to reviews by Chen *et al*. and Wibowo *et al*.[332, 333]

5.4 MESHES: SPINNINGS, KNITTINGS, AND WINDINGS

Synthetic polymer-based biomaterials are highly compatible with techniques that create meshes using for example electrospinning or fabric-based techniques like weaving, knitting or winding (figure 9).[334] Using biomaterial meshes in construct design has several advantages with respect to defined fiber alignment, adjustability, reproducibility and versatility. Synthetic polymer meshes have been readily used in the surgical arena for decades. Surgical meshes or knittings represent a group of implants mainly used for heavy-duty tissue repair, like herniations in the abdominal wall. Two major mesh concepts are distinguished, the classical concept including so-called heavy weight meshes with small pores and the newer concept including light

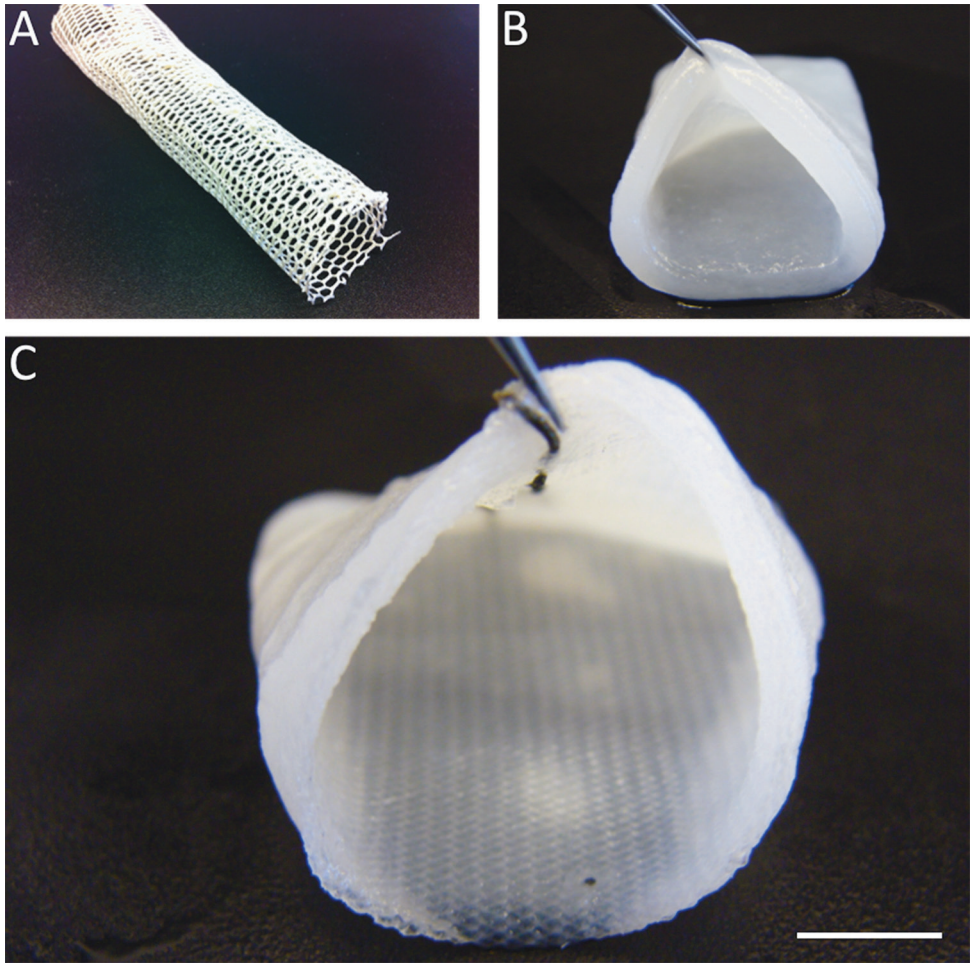

FIGURE 9: APPLICATIONS OF KNITTED POLYMERS IN COLLAGEN SCAFFOLDS.

A) A tubular knitting can be prepared from any biomaterial (in this case a synthetic polymer, polycaprolactone) that can be processed into a flexible strand. **B)** A collagen tubular collagen scaffold without reinforcement. **C)** A collagen scaffold where a biodegradable knitting was incorporated, bar represents 0.5 mm.

weight meshes with large pores.[335] Techniques used to make these meshes often require corrosive chemicals and high temperatures, which can render them unsuitable for use with natural ECM components.[336] However, in the recent years, developments in this area have resulted in several options to make meshes from more natural ECM components. Electrospinning has received bulk of the attention. It is based on the difference in (electrical) potential applied to a negative or positively charged solvent containing the polymer and a oppositely charged collector surface during extrusion.[337] During this process different fiber dimensions can be formed and deposited on wide array of surfaces to create an interconnected network or a mesh.[338] Electrospinning has been used with natural ECM components like soluble collagen, gelatin, chitosan, chitin, cellulose and starch.[339] Due to the interchangeability of the collector surface and the possibility to rotate the surface during the electrospinning process allows for control over the fiber direction in 2D meshes and 3D tubular constructs.[338,340] Other methods to create meshes from natural ECM components are often two-staged where first a fiber or strand should be extruded which can subsequently be processed into fabric-like meshes.[341]

Not all biomaterials can be extruded into a strand which is mechanically stable enough to undergo further processing.[131, 342] However, if a stable strand is formed, it can be turned into a fabric using knitting, weaving or winding. Depending on the target tissue, one can opt for making a flat mesh using knitting or weaving[343] and tubular meshes can be made using adapted knitting techniques or winding methods.[344] Different mechanical properties can be induced using different knitting, weaving and winding patterns. For example using the stockinette knitting technique, which induces anisotropic rigidity, tissues requiring particular mechanical properties in certain directions can be partially mimicked.[345]

5.5 COMPUTER CONTROLLED FABRICATION

Parallel to the rapid advancements in the field of medical imaging, where high-resolution 3D images of a defect can be easily acquired, runs the development of rapid prototyping techniques.[346] Rapid prototyping allows for the medical imaging data to be processed with computer-aided design and subsequently automatic manufacturing of three-dimensional objects layer-by-layer according to the virtual design. The

utilization of rapid prototyping in RM enables the production of 3D scaffolds with intricate geometries and very detailed structures.[347] The resulting scaffolds can be customized and made to match each patient's individual need.[348] Major rapid prototyping methodologies include 3D printing[349], multi-jet modeling[350], stereo-lithography[351], selective laser sintering[352] and fused deposition modeling[353]. These techniques are very promising for hard tissues since they are generally compatible with synthetic polymers that often require high temperatures and corrosive solvents.[354] Basically any form of lithography requires liquid-based materials which are sensitive to photo-polymerization.[355] Laser sintering and fused deposition techniques are generally based on temperature driven fusion (melting), which excludes the use of some natural ECM components. Up until now, printing techniques seem to be compatible with natural ECM components where researchers have managed to print for example collagen[356], gelatin[357], silk fibroin[358] and alginate[359]. Moreover, printing technology is also capable of producing patterns using bioactive ingredients such as growth factors.[360] In general, printing of the aforementioned biomaterials is less developed compared to the printing of synthetic polymers. Currently, problems like low resolution and low mechanical strength are being addressed. However along with the emergence of printing natural ECM components came the realization that the whole process could be conducted under aseptic/sterile conditions allowing for the addition of live cells.

Many variations have been developed but the technique is often referred to as 'bioprinting' or 'cell printing'.[361] The printing of single cells using common inkjet printer technology was useful for printing monolayers. Consequently, early efforts have focused on tissues like the skin where flat constructs were printed layer-by-layer using subsequent monolayer of cells and a hydrogel of choice.[362] A logical step forward encompassed the printing of cell aggregates or spheres. In this technique cells, in magnitude of thousands, are encapsulated in a hydrogel sphere and is subsequently printed into another hydrogel which keeps the spheres in place.[363] During culture the spheres fuse and the cells subsequently arrange in tissue like formations, subsequently circumventing the need for high-resolution printing.[364] More advanced bioprinting techniques have been developed by switching from the inkjet principle to the laser printer generation technology, known as laser-assisted bioprinting.[365] More variations on computer aided scaffold production are emerging with each method

baring its limitations. For more information regarding biomaterial printing the reader is referred to several reviews.[366, 367]

5.6 DECELLULARIZATION

Biomaterials derived from natural tissues can be referred to as decellularized matrices or extracellular matrix scaffolds. This scaffolding strategy is based on the removal of cells and cellular antigens from the allogeneic or xenogeneic tissues (figure 10). However, the ECM components, which should be well tolerated by the immune system such as collagen, elastin, glycosaminoglycans and are preserved.[368] Over the past decades many decellularization methods have been developed.[369] These cell extraction methods can impart chemical, physical and enzymatic treatments or a combination thereof. Chemical treatments are aimed at disrupting the cell membranes and intra- and extracellular structural proteins. Frequently used chemicals include detergents[370] e.g., triton x-100[371], sodium deoxycholate[372] and sodium dodecyl sulfate[373]. Also acids[374] and bases[375], hypo- and hypertonic solutions[376], organic solvents[377] and chelating agents[378] can be applied in decellularization procedures. Enzymatic treatments are mainly based on enzymes possessing protease and nuclease activity like for example ones falling within the family of trypsins[379], DNAses[380] and RNAses[381]. Physical methods to remove the cellular components can include sonication[382], high pressure[383], super-critical CO_2[384], freeze-thawing[385], agitation[386], and mechanical abrasion[387].Tissues currently under investigation include but are not limited to pericardial membrane[388], trachea[389], blood vessel[390], kidney[391], tendon and ligament[392], heart[393] and heart valve[394], cornea[395], liver[396], small intestinal submucosa[397], lung[398], nerve[399], esophagus[400], bladder[401], adipose tissue[402] and skin[403].

The advantage inherent to decellularized materials is that they can be used to generate a largely identical structure and composition of extremely complex target tissues. Depending on the method of isolation, certain components may or may not be targeted for removal. For example the use of bases like sodium hydroxide are frequently used as a virus inactivation step, however also tend to inactivate growth factors and remove GAGs. Detergents like Triton X-100 and sodium dodecyl sulfate (SDS) effectively remove nuclear remnants and cytoplasmic proteins. Decellularization methods have thus to be carefully selected to balance the scaffold requirements

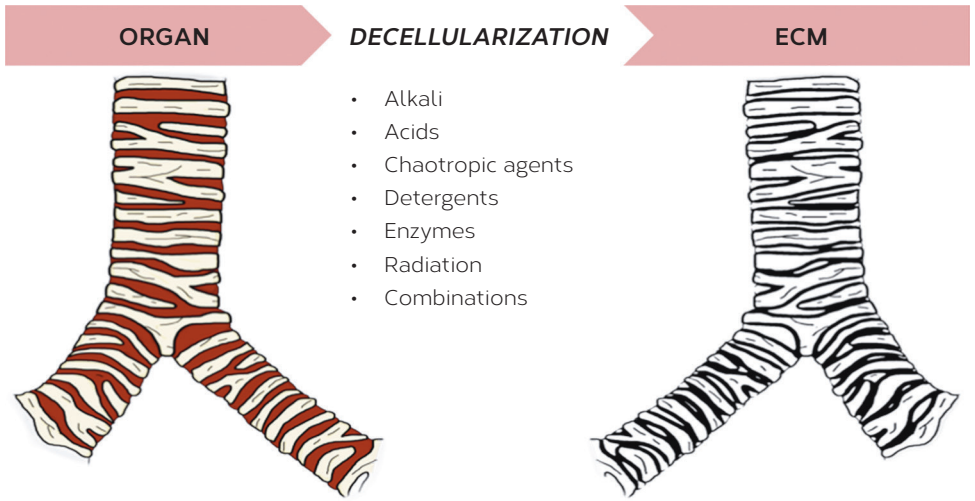

FIGURE 10: METHODS TO OBTAIN DECELLULARIZED SCAFFOLDS.

Cadaver organs, in this illustration a trachea, are exposed to a wide-array of chemical, biochemical and physical treatments aimed at removing all cellular components and unwanted proteins, effectively rendering native ECM.

with safety and efficacy. Affecting the collagen structure and GAG content may compromise mechanical integrity of scaffolds.[404] Decellularization agent remnants may have a toxicological effect on the cells, affecting the treatment efficacy.[405] Incomplete decellularization of the tissues may lead to the increased immunogenicity, ergo, increasing the possibility of eliciting an immune response.[406-408] Moreover, decellularized tissues are by nature highly variable as is the production process and it is difficult to show complete decellularization without compromising the structural integrity of the construct.

Nonetheless, decellularized tissues make up a large part of the commercially available products. Several examples of FDA approved commercially available decellularized ECM scaffolds include; AlloDerm, AlloPatch, NeoForm and Graftjacket (all human dermis), Permacol, Strattice and Zimmer Collagen Repair Patch (all porcine dermis), TissueMend (fetal bovine dermis), MatriStem (porcine bladder), CuffPatch, OaSIS, Surgisis, Restore, FortaFlex and CorMatrix ECM (all porcine small intestinal submucosa), IOPatch (human pericardium), OrthAdapt and Unite (equine pericardium), CopiOs, Lyoplant and Perimount (all bovine pericardium) and, Hancock II, Mosaic, Freestyle, Prima Plus, Epic and SJM Biocor (porcine heart valve).[369] The majority of the commercialized constructs are utilized in heart valves[409], dentistry[410], chronic wounds[411] and soft tissue reconstruction in general (hernias, burn wounds, organ slings, etc.).[412, 413] For further reading in commercially available biomaterials used in RM please see the excellent review by Keane *et al*.[200]

5.7 CROSSLINKING

Many of the described natural ECM-derived biomaterials can be subjected to a wide array of processing methods to create a 3D construct or environment. However, depending on the biomaterial type, the construct can be mechanically unstable and subject to rapid degradation. Using crosslinking techniques to enhance the mechanical and enzymatic resistance, properties of a biomaterial can be stabilized for use at physiological conditions and subsequent implantation purposes. In its essence, crosslinking is defined as the formation of bonds within or between the biomaterial subunits in question.[414] In general, the bonds formed are mostly covalent, however in

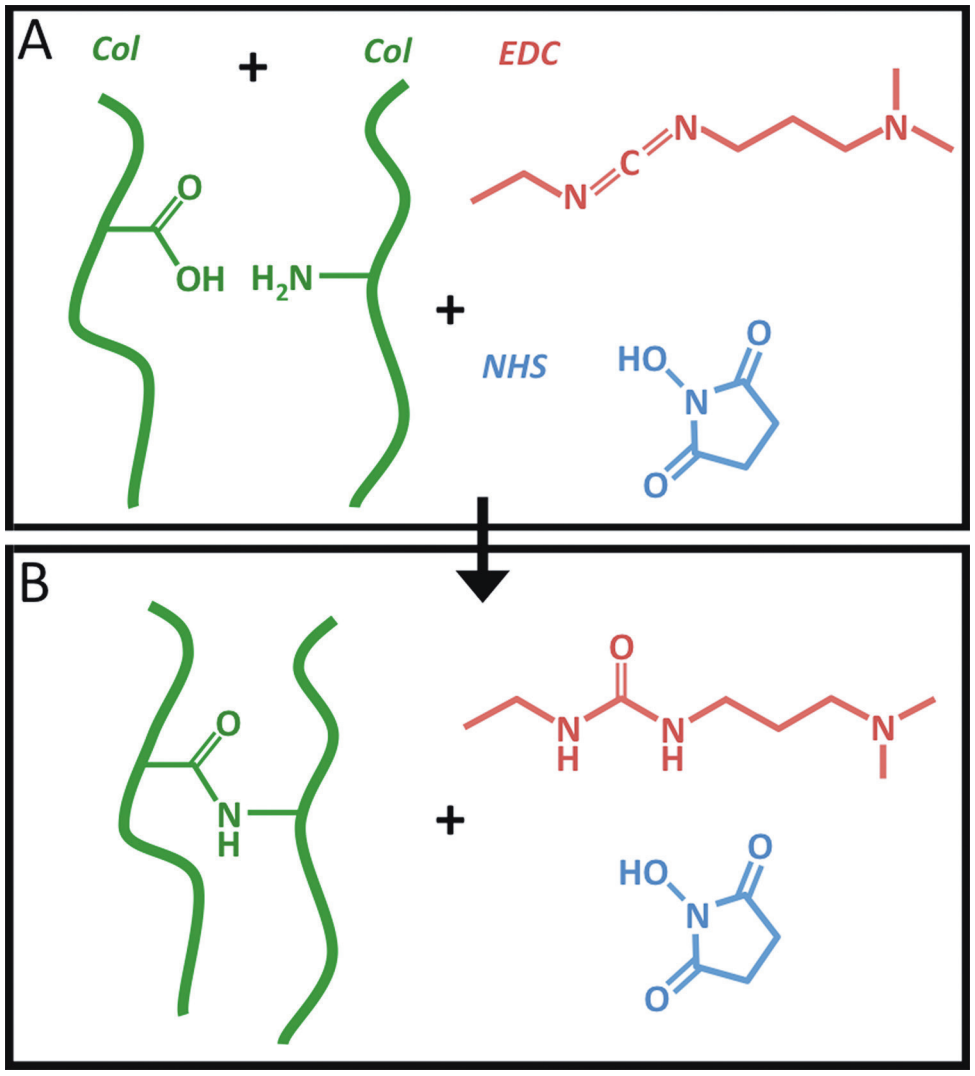

FIGURE 11: CROSSLINKING COLLAGEN USING EDC AND NHS.

A) and **B)** EDC and NHS catalyze covalent bonds between carboxylic acid and amine groups. Col: collagen. EDC: 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride. NHS: N-hydroxysuccinimide.

the case of alginate or chitosan, inducing ionic bonds using polycationic molecules can also be regarded as crosslinking.[238]

Since the majority of the natural ECM-derived biomaterials are protein or carbohydrate based, many of the developed crosslinking techniques are based on creating bonds between reactive groups like carboxylic groups and amines. Other reactive moieties include but are not limited to sulfhydryl, hydroxyl and carbonyl groups. In general there are three types of crosslinking processes: physical, chemical and enzyme-based crosslinking. Physical crosslinking methods rely on either irradiation or the use of high temperatures. Irradiation can induce free radicals that in turn react with other chemical groups near by.[415] Irradiative sources mainly refer to ultra-violet wavelengths (UV) but also to gamma and beta irradiation.[415, 416] Thermal sources in combination with high vacuum can be used to induce the dehydrothermal crosslinking which allows for formation of covalent bonds.[417] Enzymatic crosslinkers like transglutaminase can be used to enhance tensile strength and enzymatic resistance of collagen-based biomaterials.[418] One should take into account that the enzyme should also be inactivated and subsequently removed from the biomaterial in most cases. The use of enzymatic crosslinkers can eliminate the risk of inducing cytotoxic effects.[419] The largest and most diverse group of crosslinkers are the chemical crosslinkers. Glutaraldehyde along with other aldehyde-based chemicals are the most applied chemicals to crosslink protein-based biomaterials.[420] The carbodiimide family is another class of chemicals used to induce crosslinking.[421] A key feature of this method is that some carbodiimides are categorized as zero-length crosslinkers. For example EDC (1-ethyl-3-(-3-dimethylaminopropyl carbodiimide hydrochloride) directly couples primary amine groups to carboxylic groups without introducing a linker that may elicit an immune response (figure 11). A less known member of the chemical crosslinkers is the isocyanate chemical family.[422] Recently, genipin a chemical crosslinker derived from fruit extracts, has shown potential because of its low toxicity.[423] In contrast to enzymatic crosslinkers, certain chemical crosslinking techniques can potentially form toxic residues or create crosslinks and subsequent metabolic products non-native to the human body.[424, 425]

5.8 STERILIZATION OF BIOMATERIALS

Medical devices, such as ECM-based scaffolds, are made to be implanted in the patient. To avoid the risk of introducing microbes, viruses or other pathogens, sterilization is necessary. The sterilization process aims to reduce the amount of viable pathogens to an acceptable standard, since 100% sterility can never be guaranteed. The worldwide accepted standard for sterility of implantable medical devices is defined as the chance of finding a viable micro-organism in or on a medical device to be at most 10^{-6}.[426] Other pathogens like viruses, endotoxins or prions are not considered as micro-organisms, and are difficult to remove. However, biomaterials treated with sodium hydroxide can reduce the viral load and decrease the chance of prions infection.[427] In this respect, it is important to reduce the risk of introducing these pathogens during the production processes of the medical device. This can be achieved by using animal sources that originate from strictly controlled environments, specifically aimed at preventing contact with specific pathogens. For instance, for bovine-derived materials, the EDQM (European Directorate for the Quality of Medicine & HealthCare) and FDA (Federal Drug Administration, USA) recommend the use of closed-herd cattle from countries that have a low-risk for bovine spongiform encephalopathy (BSE) such as Australia or New Zealand.[428,429] Next to that, the production process of the ECM-based scaffold should be carried out in cleanroom facilities and under good manufacturing practice (GMP) guidelines. Next to these precautions, sterilization remains required to ensure acceptable sterility assurance level (SAL) values. Preferably, sterilization takes place after packaging to reduce the risk of contamination after sterilization.

Sterilization methods are based on the application of an external stimulus, such as heat, ionizing radiation or reactive chemicals, that kills or inactivates the micro-organisms, leaving the medical device functional. For medical instruments, heat sterilization (autoclaving) is often applied. However, biomaterials are not always resistant to heat, especially protein-containing materials are sensitive to high temperatures and therefore not autoclavable. Alternative sterilization methods based on chemicals or radiation are required for biomaterials. In this section the strengths and limitations of both approaches will be discussed in addition to highlighting some recent developments.

5.8.1 CHEMICAL-BASED STERILIZATION

One of most frequently applied forms of chemical sterilization is the use of ethylene oxide gas. Microbiological inactivation by ethylene oxide is based on the alkylation of hydroxylic, amine and carboxylic groups of cellular components such as DNA or proteins.[430] The ECM-based scaffolds themselves can also be affected by this powerful sterilizing agent. A recent study of Matuska and McFetridge showed that ethylene oxide caused unfavorable structural damage to decellularized collagen scaffolds.[431] In addition, ethylene oxide can change the enzymatic degradation rate of collagen, although variable results have been found, e.g. decreased degradation of dermal sheep collagen[432] versus no effect on bovine type I collagen scaffolds.[433] Oxidizing agents such as peracetic acid or hydrogen peroxide are known for their efficiency to kill microbes by denaturing proteins and disrupting cell membranes.[434] An advantage of these sterilizers, frequently used together, is that they require a much shorter cycle time compared to ethylene oxide.[435] In addition, both have a low residual toxicity and degrade into non-toxic products, namely water, oxygen and carbon dioxide.[436]

The removal of remnants of chemical sterilization from the scaffold is an important issue that should be taken seriously when applying chemical sterilization. As shown by Markowicz *et al*., hydrogen peroxide remnants in porous collagen scaffolds could still be detected after gas plasma sterilization.[437] For ethylene oxide, it is also known that remnants can be detected in medical devices after sterilization.[438] For each type of biomaterial, the bioavailability of the hazardous remnants must be evaluated.

Recent developments in the field of sterilization focus on the improvement of the delivery and removal of chemical agents in and out of the medical device. Supercritical carbon dioxide may provide an alternative means to deliver chemical sterilizers inside scaffolds. This supercritical fluid possesses physicochemical characteristics in between a gas and a liquid and therefore exhibits excellent diffusion properties.[439] It has been shown that supercritical carbon dioxide in combination with hydrogen peroxide could completely sterilize highly porous collagen sponges.[440] Another interesting physic-chemical technique is gas plasma sterilization. With this technique, a substance is brought into the gas phase under vacuum. Next, an electric field is applied on the gas resulting in the formation of reactive species, which cause microbiological inactivation. In the pure form of this technique, the gas itself has

no biocidal effect unless it is activated by the electric field.[441] Currently, gas plasma methods are mainly used in combination with a reactive compound such as hydrogen peroxide or peracetic acid.[442] Gas plasma techniques can be used at low temperatures, do not generate toxic residues and are relatively rapid.[443] In addition, penetration properties of the chemicals are superior compared to their liquid counterpart.

5.8.2 RADIATION-BASED STERILIZATION

Another conventional sterilization technique is the usage of ionizing radiation such as gamma (γ) radiation and e-beam (beta, β) radiation. γ-Radiation is generally known for its efficiency to inactivate microorganisms, however, also for its damaging effect on proteins.[433,444,445] Therefore, the scaffold and added bioactive molecules such as growth factors will always be affected. The addition of preservatives such as glucose during irradiation may reduce the side effects of γ-radiation by stabilizing the collagen molecules.[446] Next to γ-radiation, β-radiation (electron-beam) can also be used. An advantage of this technique compared to γ-radiation is that dangerous radioactive sources such as cobalt-60 can be avoided. However, the limited penetration capacity of the β-particles, make this technique less suitable for thick medical devices.[447] X-ray radiation, produced from an electron beam directed on an X-ray converter, could potentially be used to irradiate large packages. The high-energy photons damage living organisms by affecting DNA and other cellular structures. However, this technique is limited by the inefficiency of the conversion of electrons to high-energy photons, which makes the process very costly.[427] Recent developments in high-power and high-energy electron accelerators may make this sterilization approach more attractive.[448] Other than ionizing radiation, micro-waves may become applicable in the future. Shamis *et al*. reported the use of non-thermal micro-waves for microbial inactivation.[449] Unfortunately, current results with doses that do not affect the biomaterial, are not in compliance with a SAL value of 10^{-6}. Future research may clarify if this method, which avoids hazardous chemicals and radioactive sources, has potential.

5.8.3 BIOMATERIAL-SPECIFIC STERILIZATION

Despite the efficiency of reactive chemicals and radiation to inactivate microbes, damage to proteins-based scaffolds is inevitable. Moreover, current research in the field of RM is focused on creating bioactive scaffolds that contain fragile molecules such as growth factors. Therefore, recent efforts focus on developing new technologies that are as effective as previously mentioned sterilization methods while avoiding the damaging effect on the biomaterial and proteins. In addition, more environmental-friendly methods, which are easier to use without the need for severe safety precautions, would be beneficial. Sterilization will always affect the biomaterial and alter the physico-chemical and mechanical properties of the scaffold itself. In some cases, the alterations of the biomaterial can be unfavorable or non-relevant with respect to clinical use, while in other cases it can even be used to modify the scaffold to its specific needs. Andrews *et al*. showed that ethylene oxide sterilization could roughen the surface of an electrospun scaffold and thereby manipulate cell contact, phenotype or function.[450] Also for a collagen-based scaffold, it was reported that sterilization could be used to modulate characteristics to favor cellular adhesion.[431] Furthermore, sterilization can serve as a tool to modify the biodegradability of the ECM scaffold. Kawasaki *et al*. showed that gamma sterilization influenced the bioabsorption time *in vivo* for a porous hydroxyapatite/collagen scaffold.[451] For clinical applications such as biodegradable sutures, gamma sterilization is already used to adjust the bio-absorbability.[452] The sterilization method of choice is highly dependent on the type of biomaterial and the intended application of the scaffold. Consequently, no perfect method exists and researchers have to search for the best alternative for their specific scaffold and application. Furthermore, issues surrounding sterilization should be taken into account early in the developmental process to make sure that the end-product can be sterilized safely and effectively.

5.9 REGULATORY AFFAIRS

An important issue concerning biomaterials is a frequent lack of adequate attention early in the R&D process pertaining the regulatory affairs. This complicated pathway can be a huge obstacle in the translation of ECM-based scaffolds to the clinic. Although

the fundamental legislation is rather similar, most countries/continents have their own specific legislation, each with their own nuances. In a world of internationally conducted research the differences in legislation make the translational process even more difficult. Below a short overview is given on the regulatory affairs in the European Union and the United States. Acellular scaffolds generally fall within the category of medical devices according to different regulatory agencies. In Europe, medical devices are subdivided into four different classes, respectively I, IIa, IIb and III. Class III has the highest risk regarding safety issues and encompasses all implantable medical devices including scaffolds used in RM.[453] The legislation for medical devices in Europe is established by the European Commission and approved by the European Parliament (Directive 2007/47/EC). The daily execution of the regulation is controlled by so-called "Notified Bodies", which can be private companies or foundations appointed by the European Commission.[454]

A difficulty regarding regulation issues of ECM-based scaffolds is that they are often composed of components derived from animal sources. Derivatives of animal tissues may contain transmissible pathogens such as viruses, bacteria, endotoxins, and prions. Therefore, the European Union (EU) has developed extensive guidelines in order to minimize the risk of infectious disease transmission by medical implants/devices. In this respect, all relevant information about the animal source should be reported, such as -but not limited to- animal species, age, specific tissue used, country of origin, methods for health monitoring of the herd, and transportation condition. The F2027-08 document of the American Society for Testing and Materials (ASTM) provides an excellent guidance for the use of biomaterials for the purpose of RM.[455]

Next to detailed information about starting material, all details on the manufacturing process of the scaffold itself, the sterilization procedure, all test methods, test results, and acceptance criteria should be described and provided to the regulatory bodies. International Organization for Standardization (ISO) guidelines can be of great assistance for designing the manufacturing process and aim to ensure that medical devices are safe, reliable and of good quality. The regulation pathway for ECM-based scaffold is rather complex, however if (stem) cells are introduced in the scaffold, this will greatly complicate the regulation progress, since a complex viable entity is added instead of a single or a combination of compounds. For a single compound such as a growth factor, it can already be hard to predict the *in vivo* effects. For a complex cell, it will even be more complicated. In the EU, cellular scaffolds are classified under the advanced therapy medicinal products (ATMPs) and have a different and more complex legislation compared to acellular scaffolds, which are classified as medical devices.

In the US the regulatory system is rather similar to the EU. The regulation for medical devices is controlled by the Food and Drugs Administration, specifically the Center for Biologics Evaluation and Research, a center within the FDA.[456] For further information about the differences in the regulation process between the US and European union, readers are directed to a review by Vinck *et al*.[457] As the regulation and guideline issues surrounding ECM-based scaffold are very complex, most researchers do not have the proper knowledge to deal with it. To avoid foreseeable regulatory surprises, it is recommended to consult specialists in the field of regulatory issues early in the developmental process. These specialists can help researchers to avoid pitfalls and redundancy, subsequently accelerating the bench-to-bedside application of ECM-based scaffolds.

6. SUMMARY

The ECM of the target tissues/organs serves not only as a structural blueprint but also as a tool kit for tissue engineers. The natural composition of the ECM provides valuable information regarding which properties the final construct or treatment modality should mimic. By examining the role of each ECM component in the natural tissue, engineers can set priorities as pertaining to which components are most important. Major ECM components like collagen, elastin and proteoglycans play important roles in the mechanical and biological properties of the natural tissue. Due to their fibrous nature collagen and elastin have thus been used for providing mechanical strength to a construct, more so than their effects on cell behavior. Depending on the application, the state of the collagen (gelatinous, monomeric, fibrillar or fibrous) and the spatial arrangement greatly affects the mechanical properties. For molecularly defined ECM-based constructs to succeed, control over the properties of the mechanical properties (mainly the collagenous fraction) should

be increased. In most soft-tissue applications, other components like elastin and GAGs can be added to fine-tune the mechanical properties but will probably not be used in such quantity that it will take the role of collagen as the main constituent. However in load-bearing tissues such as cartilage, the water-sequestering GAGs will greatly affect the success rate of collagenous constructs by providing lubrication and shock absorbing capacity, which collagen naturally lacks. Even when the ideal mechanical properties have been attained, it will be important to ensure optimal biocompatibility, where the correct/desired cells are attracted and can subsequently adhere to the construct. Laminin, fibrinogen, fibronectin and type IV collagen are the main components of the basal membrane that regulate cell attachment and related processes. Future constructs will have to incorporate the appropriate amounts of these proteins to ensure rapid healing. Failing to attract the needed cells may lead to suboptimal formation of new ECM during the reabsorption of the construct. This in turn will ultimately lead to mechanical failure of the implant. The true challenge will be to cost-effectively incorporate (basement membrane) proteins into the constructs whilst avoiding unfavorable immune responses. More research will have to be performed to determine whether allo- or xenogeneic sources can be used. An additional concern with the use of bioactive proteins is their overall fragility. Since sterilization of a construct is unavoidable, all ingredients should be able to fulfill their respective roles after doing so. Difficulties caused by the sterilization will greatly limit the speed and overall progress of ECM-based constructs in the near future. To make future treatment modalities possible, knowledge regarding the techniques to produce different constructs and its ultrastructure in a controlled fashion needs further development. The goals of researchers in the near future would be to find a construct with an optimum between clinical outcome and overall feasibility. The feasibility and subsequent successful application of an ECM-based treatment modality mainly depends on the degree of product standardization, cost of production and overall simplicity.

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CHAPTER 02

REGENERATIVE MEDICINE FOR THE RESPIRATORY SYSTEM:

DISTANT FUTURE OR TOMORROW'S TREATMENT?

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ABSTRACT

Regenerative Medicine (RM) is a new field of biomedical science that focuses on the regeneration of tissues and organs and the restoration of organ function. Although regeneration of organ systems such as bone, cartilage, and heart has attracted intense scientific research over recent decades, RM research regarding the respiratory system, including the trachea, the lung proper, and the diaphragm, has lagged behind. However, the last 5 years have witnessed novel approaches and initial clinical applications of tissue-engineered constructs to restore organ structure and function. In this regard, this article briefly addresses the basics of RM and introduces the key elements necessary for tissue regeneration, including (stem) cells, biomaterials, and extracellular matrices. In addition, the current status of the (clinical) application of RM to the respiratory system is discussed, and bottlenecks and recent approaches are identified. For the trachea, several initial clinical studies have been reported and have used various combinations of cells and scaffolds. Although promising, the methods used in these studies require optimization and standardization. For the lung proper, only (stem) cell-based approaches have been probed clinically, but it is becoming apparent that combinations of cells and scaffolds are required to successfully restore the lung's architecture and function. In the case of the diaphragm, clinical applications have focused on the use of decellularized scaffolds, but novel scaffolds, with or without cells, are clearly needed for true regeneration of diaphragmatic tissue. We conclude that respiratory treatment with RM will not be realized tomorrow, but its future looks promising.

1. INTRODUCTION

The respiratory system comprises the airways (trachea and bronchi), the lung proper, and the respiratory muscles (e.g., the diaphragm). A number of diseases, including cancer, advanced chronic obstructive pulmonary disease (COPD), and congenital defects, require replacement or augmentation of respiratory system tissues and/or organs. However, current repair or replacement strategies are limited. Stimulating the regeneration of the patient's own tissue is an attractive alternative toward which the field of Regenerative Medicine (RM) is moving. RM is a new biomedical field of research that focuses on the regeneration of lost or damaged tissues/organs and the restoration of normal organ function.[1] At the heart of RM are two basic tissue components: (stem) cells and biomaterials (Figure 1). The field of tissue engineering, which is closely related to RM, addresses the use of scaffolds, cells, and bioactive factors to improve tissue function.[2] Although cells by themselves may give rise to new organs (e.g., transplanted bone marrow or peripheral blood stem cells), the regeneration of tissues/organs generally requires both cells and scaffolds. Consequently, respiratory RM research has addressed both entities.

The cells used in RM are derived from a variety of sources, including allogeneic, xenogenic (both immunologically unfavorable), and autologous cells. Research has focused intensely on the use of stem cells, including umbilical cord blood stem cells and amniotic fluid–derived stem cells, dedicated progenitor cells (e.g., satellite cells in the case of skeletal muscle tissue), and, recently, induced pluripotent stem cells. Cells can be used alone but can also be combined with biomaterials to produce tissue-engineered constructs. A material-based approach to RM should take into account that the composition and architecture of the extracellular matrix is specific for each organ and contains different structural and functional molecules, such as collagens, elastin, laminins, fibronectin, proteoglycans, glycosaminoglycans, and effector molecules (such as growth factors and cytokines). A biomaterial (scaffold) should preferably mimic the

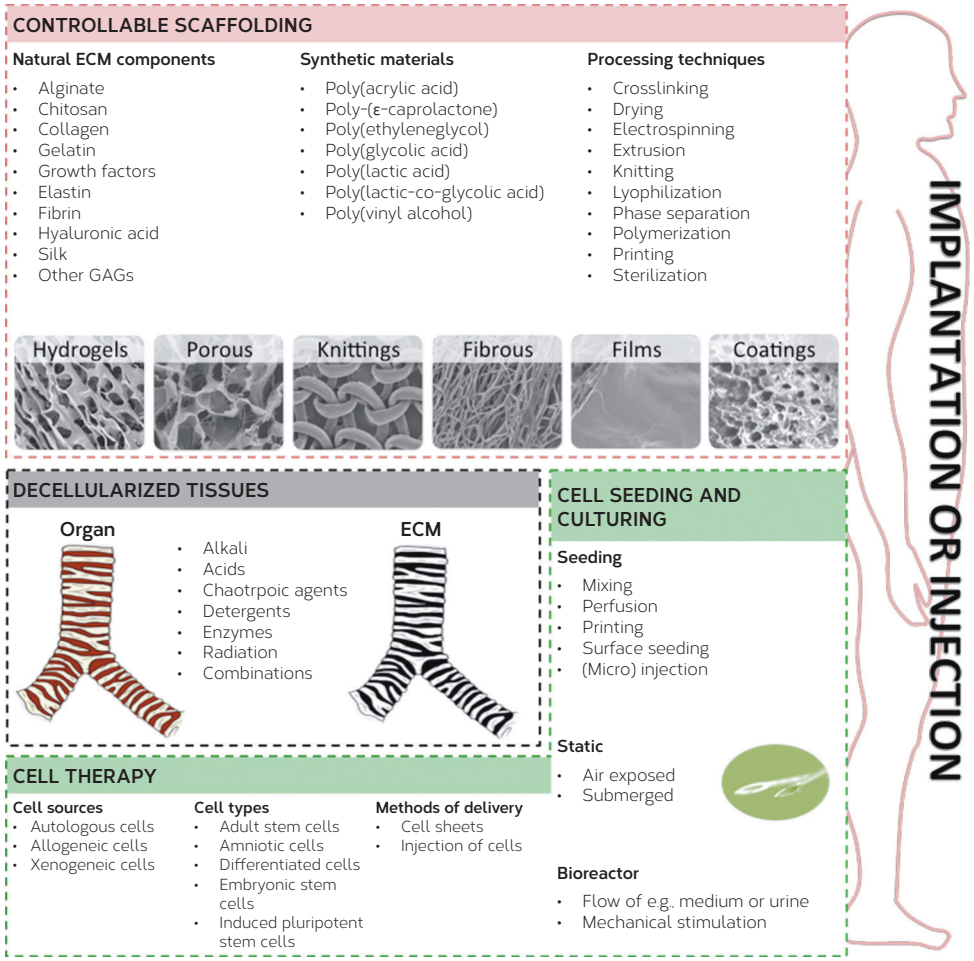

FIGURE 1: OVERVIEW OF REGENERATIVE MEDICINE APPROACHES FOR TREATING THE RESPIRATORY SYSTEM.

Cells can be used alone (cell therapy) or in combination with scaffolds prepared either from scratch (controllable scaffolding) or by decellularization of whole organs (decellularized organs). Scaffolds can also be used alone and become cellularized inside the body. ECM = extracellular matrix.

target organ's extracellular matrix and can be obtained in the following two ways: **1)** via the decellularization of organs, or **2)** via de novo construction (Figure 1). With the decellularization approach, scaffolds are generally of human, bovine, or porcine origin and can be derived from the target organ itself (e.g., using decellularized trachea as tracheobronchial constructs) or from a different organ (e.g., using decellularized porcine small intestine submucosa to close a diaphragmatic defect). These scaffolds can be used after recellularization, often requiring a bioreactor, or without cells. The main advantage of this approach is the preservation of the extracellular matrix architecture, including the vascular network. As an alternative, scaffolds can be produced from scratch using natural biological and/or synthetic materials. This approach offers the possibility of fine-tuning a number of scaffold characteristics, which includes control over the degradability rate, mechanical properties, architecture, and addition(s) of bioactive factors.

Because numerous possibilities and methods for RM approaches exist, it is necessary to establish the most suitable method for each application. Thus, this article focuses on RM approaches for treating respiratory system defects, highlighting clinical studies that have been performed and focusing on the most promising preclinical approaches. The tracheobronchial system, the lung proper, and the diaphragm are all discussed, each section beginning with a short introduction to the specific morphology involved (i.e., "what should be mimicked?") followed by the current status of the regenerative approach regarding cellular as well as biomaterial methodologies. We acknowledge and apologize for our inability to give credit to all who have contributed to the field. Definitions of terms used in this perspective can be found in the glossary (Table 1).

2. TRACHEOBRONCHIAL SYSTEM

2.1 GENERAL

The trachea is a fibromuscular tube that is 10 to 13 cm in length and 1.5 to 2.5 cm in diameter, and it is supported by C-shaped tracheal cartilage rings that are dorsally connected to smooth muscle and connective tissue fibers

(Figure 2A). The epithelium of the trachea consists of basal, ciliated, and goblet cells that are surrounded by a lamina propria (loose connective tissue with elastic fibers and seromucous glands). Inherent to its function, the tracheobronchial system is constantly in contact with the environment and, therefore, contains high amounts of immunologically active cells.[3-5] More than 40 pathological conditions affecting the airway have been identified, including congenital birth defects, cancer, trauma, infections, and stenosis, recently reviewed by Kalathur and colleagues.[6] Since the 1950s, resection, end-to-end anastomosis, and autologous tissue transplantation (aorta and esophagus) have been used to treat mild tracheal defects.[7] Extensive defects, which comprise more than 50 to 60% and 33% of the trachea in adults and children, respectively, cannot be closed by traditional means because of the lack of suitable autologous tissue.[8] Tracheal stents and T-tubes have been used to close large tracheal defects, but these methods have complications due to granulation tissue, infection, limits in graft durability, and inflammatory response.[9-11] Allo- and xenotransplantation of a (partial) trachea may be a more permanent solution for extensive lesions or end-stage disease, but concerns regarding graft rejection and immunocompatible tissue shortages exist.[12-14]

2.2 RM APPROACHES

As an alternative to existing procedures, RM approaches may avoid the complications that have frequently hindered earlier attempts. These complications include migration, dislodgement, material failure, stenosis, anastomosis failure, and the need for lifelong immunosuppression (elegantly listed by Walles in 2011).[15] Cell therapy is not the first choice, because mechanical and functional needs have to be met. The kind of biomaterial that is most often studied is generally derived from decellularized trachea and has the advantage of preserving the native extracellular matrix structure and favorable mechanical characteristics.[16] Prerequisites required for the use of biomaterials include lateral rigidity and longitudinal flexibility, airtightness, and facilitation of rapid epithelialization.

The application of RM to the tracheobronchial system has already reached the clinical stage. In 2002, a tube made of polypropylene (Marlex) mesh covered by a collagen sponge from porcine skin was used to repair the trachea of a 78-year-old woman after tumor resection, leading to full epithelialization after 2 years with no complications.[17] The first true tissue engineering approach, which was published in 2004, involved implanting (in a 58-yr-old man) a decellularized porcine jejunum patch seeded with autologous primary fibroblasts and muscle cells; this approach resulted in epithelialization and 80% cellular density after 5 days.[18] In 2008, a 30-year-old woman with end-stage bronchomalacia received a 5-cm-long left bronchus prepared from a decellularized cadaveric human trachea, which was cultured in a bioreactor for 96 hours with autologous epithelial cells and mesenchymal stem cell–derived chondrocytes.[19] It is noteworthy that the patient was not taking immunosuppressive drugs and did not develop antibodies to the graft. Lung function tests (2 months postoperative) were in the normal range, and cytological luminal scrapings revealed epithelial cells that were phenotypically identical to the ones before seeding and the presence of chondrocytes.[19] To avoid culturing cells using a bioreactor, a decellularized donor trachea seeded with autologous bone marrow stem cells was implanted directly into a 10-year-old child suffering from long-segment congenital tracheal stenosis.[6] The 7-cm-long decellularized trachea was treated with a cocktail of biologicals, including erythropoietin, granulocyte colony stimulating factor, and transforming growth factor-β, to improve stem cells recruitment, angiogenesis, and chondrocyte differentiation. The 2-year follow-up reported a functional airway, and complete epithelialization was observed after 15 months.[20]

Although results using decellularized trachea appear promising, a recent report suggests that partial collapse of constructs may occur.[21] The authors do not provide details, but mechanical stability over time is clearly an important issue. In this respect, synthetic grafts have been developed for use in tracheal repair. In 2011, a nanocomposite trachea substitute, made of polyhedral oligomeric silsesquioxane-poly (carbonate-urea) urethane (POSS-PCU), was constructed using a glass mold of the trachea of a 36-year-old patient with late-stage tracheal cancer.[22] The synthetic trachea contained U-shaped strips of the polymer (to mimic cartilage rings), was seeded with autologous bone marrow–derived mononuclear cells, and was incubated in a bioreactor for 36 hours. After reseeding and the addition of a cocktail of biologicals, the construct was implanted without the need for stenting, which is required in the case of decellularized tracheas.[23] One week after surgery, bronchoscopic analyses showed coverage of the trachea with cells. One month after the treatment, the

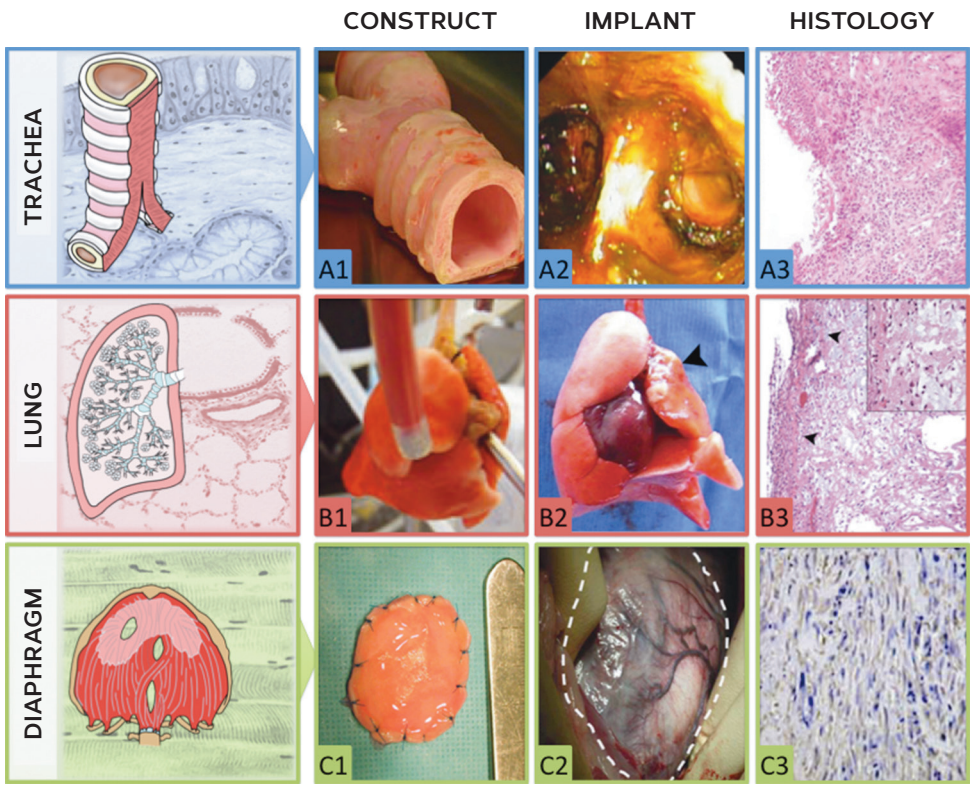

FIGURE 2: REGENERATIVE MEDICINE AND THE RESPIRATORY SYSTEM: DIFFERENT PARTS OF THE RESPIRATORY SYSTEM.

A) The tracheobronchial system (adapted and reprinted with permission[80, 81]). **B)** the lung proper (adapted by permission[81]). **C)** the diaphragm. The tissue-engineered construct used **1)** before and **2)** after implantation, and **3)** histology. **A1)** Tissue-engineered human trachea comprising a synthetic scaffold cultured with autologous human bone marrow mononuclear cells (reprinted with permission[24]). **A2)** Bronchoscopy 1 week after surgery, indicating integration with surrounding tissues (reprinted with permission[24]). **A3)** Histology 2 months after implantation, showing respiratory epithelium with mucus-secreting cells. Inflammation and ulceration were also observed (not shown) (reprinted with permission[24]). **B1)** Tissue-engineered rat lung after decellularization followed by recellularization using rat fetal lung and human endothelial cells (reprinted with permission[82]). **B2)** Macroscopic view 14 days after implantation. The arrowhead indicates the implanted lung (reprinted with permission[57]). **B3)** Histology 14 days after implantation, showing (arrowheads) increased cellularity indicative of a fibrous pleural scar (reprinted with permission[57]). **C1)** Tissue-engineered collagen-based diaphragmatic patch. **C2)** Macroscopic view 12 months after implantation in sheep, showing tendinous tissue (reprinted with permission[75]). **C3)** Histology 12 months after implantation, showing spindle-shaped cells with a (myo)fibroblast phenotype (reprinted with permission[75]).

patient was released from the hospital, and at 4 months, lung function was improved, compared with before surgery.[24] The next patient (i.e., the second-ever patient to receive such a graft) died 4 months after surgery; however, the cause of death has not been disclosed.[25]

It is clear that the search for optimal tracheal scaffolds should be intensified. Hybrid materials, which combine manmade polymers and biological materials, are likely to become the constructs of choice. A large range of basic materials, both degradable and nondegradable, are available, with a wide variety of characteristics (Figure 1). Fine-tuning these materials to the specific tracheal morphology may create the correct cues for cells to proliferate and differentiate. In this respect, fundamental studies of long-term biocompatibility are warranted.

A number of case reports on tracheal RM indicate that this approach is feasible. Each case, however, uses different protocols, including different types of cells and scaffolds. Moreover, results on long-term follow-ups and on larger groups of patients are scarce, and full disclosure of information regarding the results of patient studies has been requested.[21, 24, 26] The best protocol for tracheal RM can only be obtained by basic research regarding the fate of (stem) cells and scaffolds combined with detailed clinical trial information that includes long-term outcomes.

3. LUNGS

3.1 GENERAL

The lung is designed for the purpose of maintaining proper gas exchange (Figure 2). Starting with the primary bronchi, which originate from the trachea, there is a continuous branching of the airways. An average of 23 generations of branching is present in a human lung, the first 16 of which compose the conducting zone, whereas the remaining branches form the transition and respiratory zones. The actual gas exchange takes place in alveoli (300 million/lung), which are cup-like structures with delicate walls (alveolar septa) that facilitate gas diffusion. The barrier between alveolar air and blood in the vessels of the septa is extremely thin and mainly formed by very flat squamous cells (type I epithelial cells) that cover about 93% of

the alveolar surface. Because the lung comprises a large number of highly specialized cell types and a complex extracellular matrix, it is one of the most challenging organs in the field of RM. Nonetheless, the demand for lung tissue is high, and the treatment of major pathological conditions, such as lung cancer and pulmonary emphysema, stands to benefit greatly from the availability of RM approaches that replace affected lung tissue with new tissue. A number of current treatment modalities exist, including lung volume reduction surgery and lung transplantation, but these modalities remain challenging and have sometimes had limited success.

3.2 RM APPROACHES

Because the lung contains more than 40 different cell types and a specialized matrix structure, it should come as no surprise that regeneration of the lung is a daunting task. In essence, two main RM strategies have been applied to the lung: **1)** the cellular approach, and **2)** the combinatorial approach using cells and extracellular matrices. In addition, a more compound-based approach has been evaluated (e.g., the use of retinoic acid, a morphogen that stimulates the generation of alveoli); however, although the latter approach can be considered an RM approach, it will not be discussed here. For a recent review see Kubo *et al.,* 2011.[27]

Cell therapy has been investigated in depth (please see these recent reviews [28-32]), and initial clinical trials have been initiated or completed.[33-37] In addition to the route of delivery (intravenous or intratracheal), studies have focused on the time of delivery, safety issues associated with cell transplantation (tumor formation), and the type of cells used.[28] Clinical studies have emphasized stem cells, especially bone marrow–derived mesenchymal stromal/stem cells. Bone marrow–derived stem cells engraft into the epithelium in injured mouse lungs[38], but other studies indicate hardly any engraftment and a more immunomodulatory effect.[28] A limited number of clinical trials have been performed. In a phase I study with four patients with COPD, the use of intravenously administered autologous bone marrow mononuclear cells did not give rise to significant adverse events, as evaluated by echocardiogram and ECG and general well-being, and some lung function values were improved in the initial period (30 days) after administration.[36] Another study showed that the use of allogeneic human mesenchymal stem cells from bone marrow (Prochymal; Osiris Therapeutics Inc., Columbia, MD) in patients with myocardial infarction led to improved FEV1.[35] Interim data on a phase II study using this product in patients with COPD indicated a significantly decreased systemic inflammation in patients compared with placebo; however, pulmonary function (FEV1 and diffusing capacity of carbon monoxide) measured 6 months after intravenous administration was not significantly improved.[33,37] A phase I study on the use of autologous bone marrow–derived mesenchymal stem cells in patients with emphysema has recently been concluded[34], but results have yet to be published. Other stem cell types have been investigated but not in a clinical setting. These additional stem cells include embryonic stem cells, induced pluripotent stem cells, and placental and amniotic fluid stem cells. Studies involving these cells have recently been reviewed and the reader is referred to these excellent papers. [28, 32] Because the mode of action (e.g., engraftment into lung tissue, immunomodulatory) of the bone marrow–derived stem cells used in clinical trials is not clear, the search for resident dedicated lung-specific stem cells has intensified.[32, 39]The lung has some intrinsic regenerative capacity, notably the cuboidal type II alveolar epithelial cells, which may proliferate and differentiate into type I alveolar cells that cover most of the alveoli but are unable to replicate. Other progenitor cells in the lung include bronchiolar Clara cells and airway submucosal gland duct cells, which are capable of regenerating submucosal gland tubules and the overlying surface epithelium, and the basal cells of the airways that have been identified as stem cells in mouse trachea and human airway epithelium.[40, 41] Every dedicated stem cell described above gives rise to specific lung structures. In 2011, however, a multipotent stem cell located in the distal airways was described that, when injected into damaged mouse lung, gave rise to bronchioles, alveoli, and pulmonary vessels (including capillaries) and integrated both structurally and functionally. [42] The research, however, has been met with criticism, and a fierce debate on the existence of such a multipotent lung cell is ongoing.[43-45] The possibility of a lung stem cell capable of regenerating various lung structures is tantalizing; however, much more basic research is clearly needed.[39]

Next to cell-based therapies, scaffold-based approaches are under investigation. Because the lung is an organ with a very complex extracellular matrix that is difficult to replicate, the general strategy is to decellularize the lung, thus preserving its scaffold structure, and to recellularize it with the appropriate (stem) cells. Decellularization generally involves the use of a combination of chemical treatments (e.g., acid/

alkaline, salt solutions), detergents, and/or enzymes like trypsin and nucleases.[46-48] The extracellular matrix contains cues to direct cells to proliferate and differentiate into appropriate lung cells.[49-52] This role offers major opportunities to guide (stem) cells both directionally, using the architectural intactness of decellularized lungs, and cell biologically by providing the correct matrix substrates for proliferation and differentiation. As with tracheal RM, decellularized cadaveric human lungs may offer the appropriate microenvironment and niches for cells to form lung tissue. Cadaveric lungs should be selected very carefully, because diseased extracellular matrices may give rise to aberrant cell signaling.[53]

Decellularized lungs have been seeded with epithelial, endothelial, fetal, and/or mouse embryonic stem cells, in different combinations or alone, through the vasculature and/or airways.[50, 54-57] After recellularization and culturing in a bioreactor, lungs were implanted into rats for a period of time ranging from a few hours to 2 weeks.[50, 54, 57] Lung inflation, blood flow, oxygenation, and gas exchange were observed, but bleeding and edema were also noted.[50] In the study of Song and colleagues, which evaluated the lung 14 days after implantation (Figure 2B), athymic nude rats received a recellularized lung after left pneumonectomies.[57] The oxygenation levels of rats receiving a recellularized lung were comparable with those of animals receiving a donor lung; compared with pneumonectomy control animals, improved oxygenation was demonstrated up to day 7 but not at day 14. Histologically consolidated lung tissue, increased cellularity, and decreased aeration were observed, and a dense layer of fibroblast-like cells lining the pleural surface and limiting lung graft expansion was noted. These studies show that, although promising, this approach is still very much in its infancy. More studies on decellularization/recellularization protocols are needed, including the selection of the appropriate cell types and optimization of the culture conditions.[57]

As an alternative to scaffolds originating from decellularized lungs, the application of manmade scaffolds has been investigated. This approach avoids the decellularization step, albeit at the cost of losing the original lung matrix morphology and composition. Andrade and colleagues showed that injection of a Gelfoam sponge (pork skin gelatin) supplemented with fetal rat lung cells into lung parenchyma yielded "alveolar-like structures" at the border between the scaffold and the surrounding lung. Gelfoam sponges without cells provided a satisfactory temporary scaffold for lung cells to migrate into. However, these sponges completely dissolved after 6 months, and only a few cells could be observed in the area where the sponge had been implanted[58], apparently indicating that cells must be used for successful lung RM. Cortiella and colleagues constructed both synthetic polyglycolic acid woven mesh and pluronic F-127 hydrogel and combined both materials with somatic lung progenitor cells, in which the cells differentiated into multiple lung-specific cell types. Although *in vitro* experiments showed lung-specific markers for Clara cells, pneumocytes, and respiratory epithelium and organization into lung-like structures, implantation of the polyglycolic acid–based constructs with somatic lung progenitor cells into immunocompetent hosts provoked a large immune response.[59]

The data presented above clearly indicate that, although RM of the lung is very much in its infancy, the field has seen major progress in the last 5 years. A combination of (stem) cells and scaffolds obtained through decellularization protocols seems the most promising strategy at present. However, given the variability in the decellularization process, a next step could be the modular three-dimensional production of lung scaffolds from scratch using purified (biological) components, such as collagen, elastin, and growth factors that reflect the lung's architecture and provide signaling cues that cause (stem) cells to home, proliferate, and differentiate into lung tissue.

4. DIAPHRAGM

4.1 GENERAL

The respiratory muscles are primarily made up of the diaphragm and the intercostal muscles. The diaphragm is a musculotendinous septum consisting of a central tendon plate to which muscle fibers are attached. It contains three major openings allowing passage of the esophagus, vena cava, and aorta (Figure 2C). Skeletal muscle fibers are radially oriented, and stem cells (satellite cells) reside beneath the basal lamina. Mesothelium covers both the abdominal and thoracic sides of the diaphragm and is known as the peritoneum and parietal pleura, respectively.

Whereas intercostal muscle defects in need of extensive reconstruction are rare, diaphragmatic defects are observed more frequently. Diaphragmatic defects can originate through trauma or resections, for instance in cases of neoplastic growth, or by congenital diaphragmatic hernia, an inborn disorder that affects 1 in 2,500 to 4,000 births.[60] Diaphragmatic defects can often be repaired primarily, but in the case of large defects, suturing of the wound edges is not possible due to lack of tissue. In these cases, a patch is required to close the defect. Approximately one-half of the infants undergoing repair for congenital diaphragmatic hernia require patches (Congenital Diaphragmatic Hernia Registry).[61] Lasting repair can be difficult to achieve, because the patch often does not grow along with the child, leading to a situation in which patch disruption (reherniation) occurs. In general, patch repair is performed using prosthetic patches (including polytetrafluoroethylene [PTFE], Silastic, Dacron, and Gore-Tex-Marlex composites). Although some groups have been successful with this approach[62-64], reherniations after closure with PTFE patches have been frequently observed.[65, 66] Moreover, chest wall deformities and scoliosis have been reported.[67] Next to prosthetic patch repair, several methods for muscle flap repair have been used successfully, although abdominal bulging can occur, and children on extracorporeal membrane oxygenation may experience bleeding problems from such an invasive procedure and the anticoagulants required.

4.2 RM APPROACHES

A main advantage of using RM methods to treat congenital diaphragmatic defects is that the construct applied may grow with the child. Ideally, new skeletal muscle fibers should form, adopting a radial orientation. The use of absorbable extracellular matrix patches, including decellularized porcine skin (Permacol), human skin (Alloderm), and porcine small intestinal submucosa (SIS), has been investigated. Although preclinical studies have indicated that acellular small intestinal submucosa could be a successful alternative to PTFE,[68, 69] SIS patches did not lower recurrence rates in a clinical setting,[65] possibly because of rapid degradation of the construct. Other constructs, including molecularly defined porous collagen scaffolds, have been evaluated preclinically. In a rat model, such scaffolds have shown potential to support muscle ingrowth and prevent reherniation.[70] Collagenous scaffolds with a radial pore structure, which is anticipated to direct muscle growth, have also been constructed.[71] A collagen-poly-lactic-co-glycolic acid (PLGA)-hybrid scaffold model effectively closed a defect of 1.5 × 1 cm^2, and vascularization and fibrous tissue formation were observed.[72] Seeding bone marrow–derived mesenchymal stem cells did not improve the outcome. Conconi and colleagues showed that, in a rat model, decellularized diaphragmatic matrices seeded with muscle precursor cells led to longitudinally aligned muscle fibers, whereas acellular constructs led primarily to fibrosis.[73]

In 2001, Fauza and colleagues were the first to use fetal cells, with the advantage of having a construct present at birth for the child.[74] Myoblasts were harvested from fetal lambs, cultured in a collagen hydrogel under radial tension, placed between layers of acellular small intestinal submucosa, and implanted in young lambs. Sixty days after implantation, the cellularized constructs contained muscle-like tissue, with few eventrations. In a similar study[75], the fetal myoblasts quickly lost their myogenic phenotype. To avoid the ethically challenging use of fetal muscle tissue, the use of mesenchymal amniocytes harvested from amniotic fluid has been investigated. Compared with both equivalent acellular and fetal myoblast based grafts, collagenous constructs containing mesenchymal amniocytes led to improved outcomes with respect to occurrences of reherniation and biomechanical characteristics.[75-77] Autologous tendon-like tissue was formed. Given the difficulties involved in the regeneration of skeletal muscle tissue, this approach may be the most promising for initial clinical RM studies for treating congenital diaphragmatic hernia. A preclinical regulatory validation study has been performed, and "clinical trials of this methodology appear amenable to Food and Drug Administration consent and are arguably justified in view of the persistently poor results of current treatment modalities for larger diaphragmatic defects".[78]

As we await the first clinical trials, at present, the most successful construct addresses the regeneration of the diaphragmatic tendon, not the skeletal muscle. For optimal functionality, however, diaphragmatic muscle regeneration, including innervation, is essential. A major challenge results from the fact that muscle cells in tissue-engineered constructs are generally refractory to differentiation into functional units. A recent study of the functional recovery of denervated muscle after transplantation of nerve tissue underscores the importance of neurogenic elements

and suggests that adding neurotrophic growth factors to scaffolds may stimulate muscular development in constructs used to regenerate the diaphragm.[79]

5. FUTURE PROSPECTS

Reports on RM for the respiratory system are increasing both in number and quality, and our understanding of stem cells and extracellular matrices has now been extended to the respiratory system. Tracheobronchial RM has reached the stage of clinical trials, and promising initial results have been observed when the patient's cells are combined with decellularized extracellular matrices/synthetic manmade scaffolds. More extensive clinical trials are now needed to firmly establish the application of RM to the tracheobronchial system.

RM approaches for the regeneration of lung tissue proper have not reached the clinical stage. Clearly, regeneration of lung tissue requires stem cells. Major progress has been made in identifying lung-resident stem cells. However, it will be a challenge to expand such dedicated stem cells in culture to amounts suitable for clinical applications. Application of bone marrow–derived stem cells is already in early clinical trials and appears to be well tolerated and safe. However, the use of RM based on extracellular matrices/scaffolds is in its infancy. Rigid protocols should become available to ensure optimal and reproducible de- and recellularization procedures. The use of manmade scaffolds produced from scratch should be pursued, because this strategy allows the construction of personalized scaffolds, with 3D structures and dimensions specific for individual patients, and may increase reproducibility. Novel technologies capable of combining molecularly defined biomaterials with computer precision, such as electrospinning, soft lithography, and 3D printing, hold promise in this respect.

Embryonic scaffolds, which contain cues that induce (stem) cells to generate lung tissue, may pay off in the end; however, at the moment, virtually no research is being conducted along this line. Extracellular matrix–based materials have been used for a number of years to close congenital diaphragmatic hernia; however, their success is not without limitations. Clinical trials for tissue-engineered constructs appear near[77], and we hope that these trials will answer critical questions regarding diaphragm RM, especially with respect to the regeneration of skeletal muscle fibers and correct innervation.

In conclusion, in the last decade, the field of RM has entered the arena of the respiratory system. Research on lung stem cells is now in full swing, but research regarding extracellular matrices, which are of eminent importance in pulmonary functioning, is lagging behind. In addition, issues like cost effectiveness, practicality, and regulatory guidelines are still being addressed. It will likely be another 5 to 10 years before large clinical trials for the tracheobronchial system are initiated, and the clinical trials of lung RM are expected to take at least twice this long. Thus, although we may not have to wait until the very distant future to realize applications of RM to the respiratory system, they are not expected tomorrow.

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LIST OF FIGURES & TABLES

TABLE 1: GLOSSARY OF TERMS AND DEFINITIONS.

Allogeneic	From a different individual from the same species.
Autologous	From the same individual (donor and receiver are the same person).
Biomaterial	Material that has been engineered to take a form which, alone or as part of a complex system, is used to direct, by control of interactions with components of living systems, the course of any therapeutic or diagnostic procedure (definition journal 'Biomaterials').
Cell therapy	Use of cells to treat a disease.
Decellularization	Removal of cells and cellular components from tissue.
Embryonic stem cell	Pluripotent stem cells from the inner cell mass of a blastocyst.
Extracellular matrix	Matrix of macromolecules outside cells, providing structural support and biological cues.
Induced pluripotent stem cell	Pluripotent stem cell artificially derived from a non-pluripotent (adult) cell by forced expression of a limited number of specific genes.
Mesenchymal stem cell	Multipotent stem cells that can differentiate into a variety of cell types, including osteoblasts, myocytes, chondrocytes, fibroblasts and adipocytes.
Regenerative Medicine (RM)	Scientific medical field aiming to regenerate (rather than repair) tissues and organs, in order to restore or establish normal function; using (stem) cells and/or biomaterials.
Scaffold	Three-dimensional structure capable of supporting and instructing cells, facilitating tissue formation.
Stem cell	Cell capable of forming different specialized cell types and self renewal.
Tissue engineering	Interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ, using *in vitro* engineered constructs consisting of cells, biomaterials and/or bioactive factors.
Xenogenic	From an organism of a different species.

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CHAPTER 03

SEAMLESS VASCULARIZED LARGE-DIAMETER SCAFFOLDS FOR ESOPHAGEAL REGENERATIVE MEDICINE:

TUBULAR COLLAGEN SCAFFOLDS REINFORCED WITH POLYMER KNITTINGS

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ABSTRACT

A clinical demand exists for alternatives to repair the esophagus in case of congenital defects, cancer, or trauma. A seamless biocompatible off-the-shelf large-diameter tubular scaffold, which is accessible for vascularization, could set the stage for Regenerative Medicine of the esophagus. The use of seamless scaffolds eliminates the error-prone tubularization step, which is necessary when emanating from flat scaffolds. In this study, we developed and characterized three different types of seamless tubular scaffolds, and evaluated *in vivo* tissue compatibility, including vascularization by omental wrapping. Scaffolds (luminal Ø ~1.5 cm) were constructed using freezing, lyophilizing, and crosslinking techniques and included **1)** single-layered porous collagen scaffold, **2)** dual-layered (porous and dense layer) collagen scaffold, and **3)** hybrid scaffold (collagen and incorporated polycaprolacton knitting). The latter had an ultimate tensile strength comparable to a porcine esophagus. To induce rapid vascularization, scaffolds were implanted in the omentum of sheep using a wrapping technique. After 6 weeks of biocompatibility, vascularization, calcification, and hypoxia were evaluated using immunohistochemistry. Scaffolds were biocompatible, and cellular influx and ingrowth of blood vessels were observed throughout the whole scaffold. No calcification was observed, and slight hypoxic conditions were detected only in the direct vicinity of the polymer knitting. It is concluded that seamless large-diameter tubular collagen-based scaffolds can be constructed and vascularized *in vivo*. Such scaffolds provide novel tools for esophageal reconstruction.

1. INTRODUCTION

Esophageal replacement so far has remained well beyond the horizon of our clinical armamentarium, but once feasible, this strategy would become invaluable in case of several acquired and congenital esophageal conditions, including atresia, (pre)malignancy and resistant strictures, and esophageal cancer. Congenital esophageal malformations have an occurrence rate of 1:2500 live births. In cases where primary anastomosis is not possible, complex reconstruction is required. Applied techniques utilize autologous grafts, for example, esophageal lengthening techniques (flap, gastric division, and spiral myotomy), transmediastinal thread, and esophageal substitution (colonic, jejunal or gastric interposition, and gastric tube esophageal substitution).[1, 2] Post-operative complications of the esophagus are inherently associated to such reconstructions, including dysmotility and dysphagia, anastomotic stricture formation, anastomotic leakages, gastroesophageal reflux, and recurrent tracheoesophageal fistula.[3–9] Moreover, harvesting of autologous grafts can lead to severe complications, including anastomotic leakages, enteric fistulae, bowel obstruction, prolonged episodes of ileus, life-threatening infections, and/or intestinal failure.[10, 11] Esophageal cancer is the eighth most common cancer worldwide, and it is the sixth most common cause of death from cancer with 406,000 deaths (5.4% of the total).[12]

The ideal surgical procedure would lead to the restitution of a functional esophagus without requiring the use of other autologous gastrointestinal tissue. Autologous tissue may be replaced using materials and techniques generated in the field of Regenerative Medicine (RM).[13] RM aims at regenerating diseased, damaged, or missing tissues by creating biological equivalents through the supplementation of cells, scaffolding materials, and bioactive components or a combination thereof.[14] Autologous stem cells in combination with scaffolds that mimic the structure of the hollow organ in question, while simultaneously sustaining tissue regeneration, could

lead to improved treatment options.[15, 16] Recently, Badylak *et al*. provided a clinically oriented review regarding the status of RM of the esophagus, where the authors seem to prefer a decellularized tissue approach in combination with autologous cells.[17] Kuppan *et al*. and Tan *et al*. extensively reviewed the attempted tissue engineering (TE) interventions for esophageal disorders, where both groups concluded that a suitable scaffold has yet to be made.[18, 19] Recently, Shen *et al*. extensively reviewed the materials and scaffolding techniques that have been suggested for esophageal TE.[20] Moreover, Totonelli *et al*. reviewed the different *in vivo* implantations of acellular matrices for esophageal reconstruction and concluded that several major issues need to be addressed before a successful therapy can be developed, including vascularization.[21] Adequate nutrient and sufficient oxygen supply by the vascular network to a graft is one of the main challenges within the field of RM.[22] A promising procedure to vascularize acellular scaffolds is *in vivo* omentum wrapping.[23, 24] After a predetermined time to permit vessel ingrowth and/or graft remodeling in the "omental bioreactor," the vascularized scaffolds can be used as a pedicle graft for RM.

A number of RM strategies and several biomaterials have been used in gastroenterology.[2, 22, 25–27] A combination of decellularized porcine small intestinal submucosa (SIS) and mesenchymal stem cells was used to repair esophageal defects in dogs with some success.[28] However, the defect was partial being 50% of the circumference, hampering extrapolation of the results to full circumference defects. Interestingly, Doede *et al*. attempted to repair 4 cm full circumference esophageal defects using acellular SIS in piglets but observed severe stenosis in most animals.[29] Paramount to the success of RM is the ability to make patient-specific scaffolds, which not only regenerate the tissue but also are reproducible in quality. Decellularized tissues are, by nature, highly variable as is the production process, and incomplete decellularization can have severe consequences on the regeneration as reviewed by Keane *et al*.[30] Substituting decellularized cadaver tissue by scaffolds prepared from scratch using purified biological materials such as collagen can circumvent these issues. The advantages of molecularly defined materials have previously been demonstrated.[31–35] For RM of the esophagus, such scaffolds should preferably be made in a tubular shape, thus eliminating the error-prone tubularization step, which is necessary when emanating from flat scaffolds. As a proof of principle, in the present study, we aimed at developing a number of vascularized and seamless, tubular scaffolds based on purified collagen fibrils, with adequate mechanical strength that could in the future be used in esophageal RM.

2. MATERIALS AND METHODS

2.1 TYPE I COLLAGEN FIBRILS

Highly purified type I collagen fibrils were obtained as described.[36] Briefly, bovine Achilles tendons were pulverized (0.5 mm) in a universal cutting mill (Pulverisette19; Fritsch GmbH) under liquid nitrogen-cooled conditions. The purification process included washings with aqueous solutions of NaCl and urea, diluted acetic acid, acetone, and demineralized water. After purification, type I collagen fibrils were frozen, lyophilized, and stored under vacuum at –20°C.

2.2 POLYMER SYNTHESIS

Polycaprolactone (PCL), a biodegradable polyester, was synthesized by ring-opening polymerization of ε-caprolactone monomer (1 kg of monomer was purified by distilling twice over CaH_2 using 3 mL of a 0.61 g/mL solution of $Sn(Oct)_2$ in toluene as polymerization catalyst). The monomer and catalyst were mixed thoroughly, and the mixture was kept at 110°C for 2 days for polymerization.

2.3 POLYMER THREAD AND KNITTING

PCL was spun into a monofilament using lab-scale melt spinning. For this, PCL was first dried overnight at 40°C under vacuum, and placed into a thermostated mold (80°C), which was fitted with a spinneret of 1.1 mm. Before melt spinning, the polymer was compressed under nitrogen atmosphere to remove voids and air. Subsequently, the mold temperature was raised to 180°C, and the melt spinning was carried out by extruding the PCL through the spinneret into a monofilament directly into an ice bath (at 2°C). The monofilament was collected on a bobbin, and the filament was drawn six times at 40°C to introduce orientation, crystallinity, and mechanical

strength as preparation for knitting. The polymer knitting was prepared from the filament using knitting machinery (Lawson Hemphill) with a 29-Gauge needle.

2.4 SCAFFOLD CONSTRUCTION

Three different tubular scaffolds were prepared (Figure 1): two types consisting of collagen only, and one containing an additional PCL polymer knitting.

2.4.1 SINGLE-LAYERED TYPE I COLLAGEN SCAFFOLD (FIGURE 1A)

Type I collagen was swollen in 0.25 M acetic acid at a concentration of 0.67% (w/v) for 16 h at 4°C. The obtained suspension was homogenized using a Potter–Elvehjem homogenizer (Louwers glass and Ceramic technologies) with an intervening space of 0.35 mm (all at 4°C) using ~10 strokes. The suspension was deaerated by centrifugation at 160 g for 30 min at 4°C, and it was subsequently incubated under vacuum in a desiccator for 30 min at room temperature (RT). Next, type I collagen suspension was placed in a polystyrene mold (Ø 28 mm; Falcon, BD, Bioscience), and a stainless steel mandrel (Ø 15 mm) was positioned in the center of the mold. Casted molds were frozen for 4 h at −20°C (in a custom-made aluminum block) and subsequently lyophilized (500II sublimator; Zirbus). Tubular collagen scaffolds thus obtained were crosslinked by means of N-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS).[37] Briefly, tubular scaffolds were pre-incubated for 30 min at RT in 50 mM 2-morpholinoethane sulfonic acid (MES, pH 5.0) (USB) containing 40% (v/v) ethanol. Next, the scaffolds were placed for 4 h at RT in 33 mM EDC (Fluka Chemica) and 6 mM NHS (Fluka Chemica) in MES buffer containing 40% (v/v) ethanol. Scaffolds were washed with 0.1 M Na2HPO4, 1 M and 2 M NaCl, and demineralized water while shaking, washed with 70% (v/v) ethanol, and stored at −20°C.

2.4.2 DUAL-LAYERED TYPE I COLLAGEN SCAFFOLD (FIGURE 1B)

To reinforce the single-layered collagen scaffold, a second was added to form dual-layered scaffolds. First (non-crosslinked) single-layered type I collagen scaffolds were made as described earlier. Next, the tubular collagen scaffold's wall was evenly compressed around a stainless steel mandrel to about 0.2 mm wall thickness, applying a rolling motion on a clean and smooth surface, which was repeated eight times. The compressed tubular collagen scaffold was removed from the mandrel and crosslinked by means of EDC/NHS (and washed) as described earlier, but without performing the MES/ethanol pre-incubation step to prevent re-swelling of the compressed layer. To apply a second porous layer, the stainless steel mandrel was put back into the crosslinked and compressed tubular collagen scaffold, and was subsequently incubated in 2 M acetic acid for 1 h at RT. Then, the mandrel with scaffold was placed in a mold containing deaerated and homogenized 0.67% (w/v) type I collagen suspension in 0.25 M acetic acid. Molds were frozen for 4 h at −20°C (in the custom-made aluminum block) and subsequently, lyophilized. After lyophilization, EDC/NHS crosslinking (with MES/ethanol pre-incubation) was repeated as described earlier.

2.4.3 HYBRID SCAFFOLD (FIGURE 1C)

Another method to reinforce porous tubular scaffolds is the addition of man-made polymers. For the construction of tubular hybrid scaffolds, the knitted PCL polymer sheet was wrapped around a stainless steel mandrel such that the sheet's back (Figure 2A, B) faced the mandrel, and the knitting direction (KN) was orientated circumferentially. The polymer sheet was sutured into a tubular form using 4-0 Perma-hand silk running sutures and five evenly spaced triple square knots (Ethicon). The tubularized knitting, with mandrel was incubated in 0.25 M acetic acid for 1 h at RT and subsequently, placed in a mold containing deaerated and homogenized 0.67% (w/v) type I collagen 0.25 M acetic acid suspension. The casted molds were frozen for 4 h at −20°C and lyophilized. After lyophilization, EDC/NHS crosslinking was performed as described earlier.

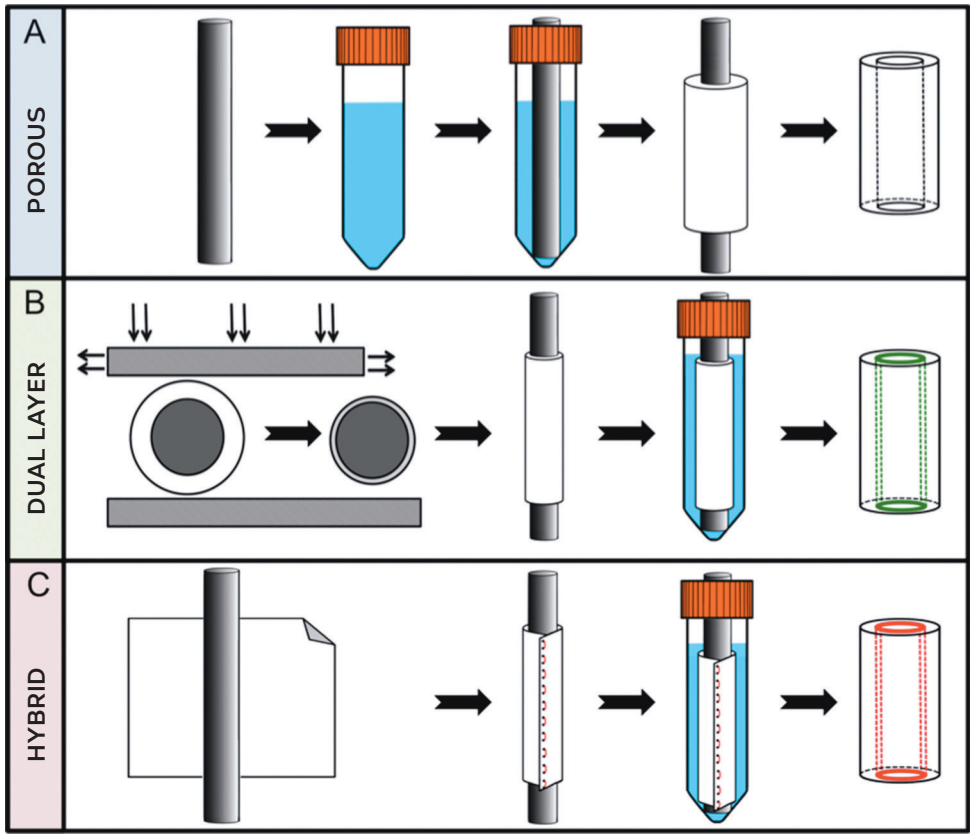

FIGURE 1: SCHEMATIC REPRESENTATION OF THE MANUFACTURING PROCESS OF THREE TYPES OF LARGE-DIAMETER COLLAGEN-BASED TUBULAR SCAFFOLDS.

Scaffolds were constructed using stainless steel mandrels (Ø=15mm) and polystyrene tubes. **A)** Single-layered porous scaffold. **B)** Dual-layered semi porous scaffold, made by compressing a porous scaffold around a mandrel (followed by crosslinking), and insertion into a new collagen suspension. **C)** Hybrid scaffold consisting of a collagen and a polycaprolactone knitting, which was tubularized using sutures. Collagen was cast in the polystyrene molds, after which the mandrels were inserted and secured using a cap with a centering mechanism. The tubes were subsequently frozen in a custom-made aluminum block at -20°C. Implantable disinfected scaffolds were obtained after removal from the molds and mandrels and subsequent crosslinking and ethanol wash steps.

2.5 CHARACTERIZATION

2.5.1 SCANNING ELECTRON MICROSCOPY (SEM)

SEM was used to analyze the morphology and structure of the tubular scaffolds. Samples were lyophilized, or when seeded with cells, dehydrated with an ascending series of ethanol solutions, and critically point-dried (Polaron, Quorom Technologies, Rignmer, United Kingdom). Next, samples were fixed on a stub with double-sided carbon tape and sputtered with an ultrathin gold layer in a Polaron E5100 Coating System. Examination was performed in a JEOL SEM 6310 apparatus (JEOL Ltd, Tokyo, Japan) with an accelerating voltage of 15 kV.

2.5.2 CONTENT OF AMINE GROUP AFTER CROSSLINKING

The degree of crosslinking was evaluated by the loss of primary amine groups on crosslinking that was measured by a reaction with trinitrobenzene sulfonic acid.[38]

2.5.3 ULTIMATE TENSILE STRENGTH (UTS)

A validated device was used to measure mechanical strength.[39] Tubular scaffolds were cut longitudinally and placed in between two clamps. The force applied to rupture was digitally recorded by a computer. All measurements were performed under the same conditions at an elongation speed of 2.9 mm/min, with a sample length of 8 mm and pre-incubation of scaffolds for 16 h in demineralized water (wet conditions). Measurements were repeated depending on the amount of available material (single layered scaffold n=8; dual-layered scaffold n=9; hybrid scaffold n=9; knitted synthetic polymer n=4 (both in the KN and perpendicular to the knitting direction [PKN]). As a reference, adult porcine esophagus (n=6, transported in 0.9% NaCl) was used.

2.6 *IN VIVO* VASCULARIZATION AFTER OMENTUM WRAPPING

All procedures were performed according to the Institute of Laboratory Animal Research (ILAR) guide for laboratory animals.[40] This study was approved by the

Animal Ethics Committee, Ministry of Science and Research, Vienna, Austria (BMWF-66.010/0015-II/10b/2009). To study the *in vivo* biocompatibility, the tubular scaffolds were implanted in sheep by omental wrapping (Mountain sheep, ♀, and age ~1 year). Anesthesia was induced by an intravenous injection of propofol (5 mg/kg; Pharmacia, AstraZenaca) followed by tracheal intubation. The sheep were maintained on general anesthesia with 1.5% (v/v) isoflurane (35%O2 Isoflurane Nicholas Piramal; Nicholas Piramal India Ltd.). For analgesia, flunixin (2 mg/kg; Schering Plough, Sante Animal) and sufentanil (4 µg/kg; Janssen Cilag) were given intravenously followed by a maintenance dosage sufentanil of 2 µg/kg/h. Heart rate, temperature, oxygen saturation, and carbon dioxide concentration of the expired air were monitored.

The abdomen was shaved, cleaned, and aseptically prepared with iodine. After a low midline laparotomy, the omentum was exteriorized. Disinfected scaffolds were stented on endotracheal tube stents (Rusch Flexi-set PVC Endotracheal tube; Rusch AG) and enwrapped within an omentum fold. Each sheep received one of each scaffold type. Sutures were placed along the omentum fold to prevent unwrapping and to fix the tubular scaffolds before the omentum was interiorized. Subsequently, the wound was closed: The abdominal wall and skin were closed in three layers using 2-0 vicryl continuous sutures for the peritoneum, 1-0 vicryl for the fascia, and 1-0 polyester sutures for the skin. Depomycin (20 mg/kg, subcutaneous; Intervet) was administered preoperatively and maintained postoperatively for 3 to 5 days via intramuscular injections. To provide postoperative analgesia, buprenorphine (10 µg/kg, intravenous; Schering Plough) and flunixin (2 mg/kg, intramuscular) were given for 3 days. After 6 weeks, the animals were sacrificed after anesthesia by administration of 10 mL 10% KCl intravenously. The tubular scaffolds with omentum tissue were explanted, fixed (4% formalin), and paraffin embedded for immunohistological analysis.

2.7 HISTOLOGY

Paraffin sections were cut (5 µm), and hematoxylin and eosin (HE) staining was used to visualize the general morphological features of the implanted scaffolds. Masson Trichrome (MT) staining was applied to visualize collagen (green), cytoplasm (red), and cell nuclei (black) in explanted scaffolds. Possible calcification was analyzed by Von Kossa staining.[41]

2.8 IMMUNOHISTOLOGY

Paraffin sections were cut (5 µm). Immunohistochemical staining was performed in type IV collagen and hypoxia-inducible factor 1-alpha (HIF-1α). In brief, sections were deparaffinized by xylene and hydrated in a degrading series of ethanol. Antibody retrieval was performed by boiling in 10 mM sodium citrate pH 5.5 for 10 min (type IV collagen and HIF-1α). After blocking endogenous peroxidase activity with 0.3% H2O2, sections were incubated for 16 h at 4°C with polyclonal rabbit anti-human type IV collagen (1:100; Abcam) or monoclonal mouse anti-human HIF-1α (1: 500; Abcam). After PBS washings, sections were incubated for 1 h with appropriate biotinylated secondary antibodies (1: 400, Vectastain Elite ABC-kit; Vectorlabs). After washings with phosphate-buffered saline (pH 7.4), sections were developed with 3,3′-diaminobenzidine (DAB) as a chromogen. Sections were dehydrated in an increasing series of ethanol and mounted with entellan.

3. RESULTS

3.1 SCAFFOLD CONSTRUCTION AND MORPHOLOGY

All tubular scaffolds with a length of 5 cm displayed a homogenous sponge-like appearance (Figure 2). Freezing collagen suspensions in an aluminum block facilitated a homogeneous pore structure. For the construction of dual-layered scaffolds, non-crosslinked single-layered scaffolds were evenly compressed and directly submerged in the crosslinking solution, causing preservation of the compressed morphology. Subsequent incubation in 2 M acetic acid facilitated adhesion of the porous collagen layer. A similar protocol for the hybrid scaffolds promoted collagen-polymer interaction, resulting in better adherence of the two layers using pre-incubation in 0.25 M acetic acid. The lumen of the single- and dual-layered scaffolds (both collagen only) collapsed (Figure 2C, F). In contrast, the lumen of hybrid scaffolds remained open (Figure 2H). The PCL polymer structure was prepared by knitting the thread into sheets according to a ‘stockinette’ structure (Figure 2A and B). The knitting, and therefore the hybrid scaffold as well, displayed anisotropic behavior while handling, namely elasticity in the KN and rigidity PKN.

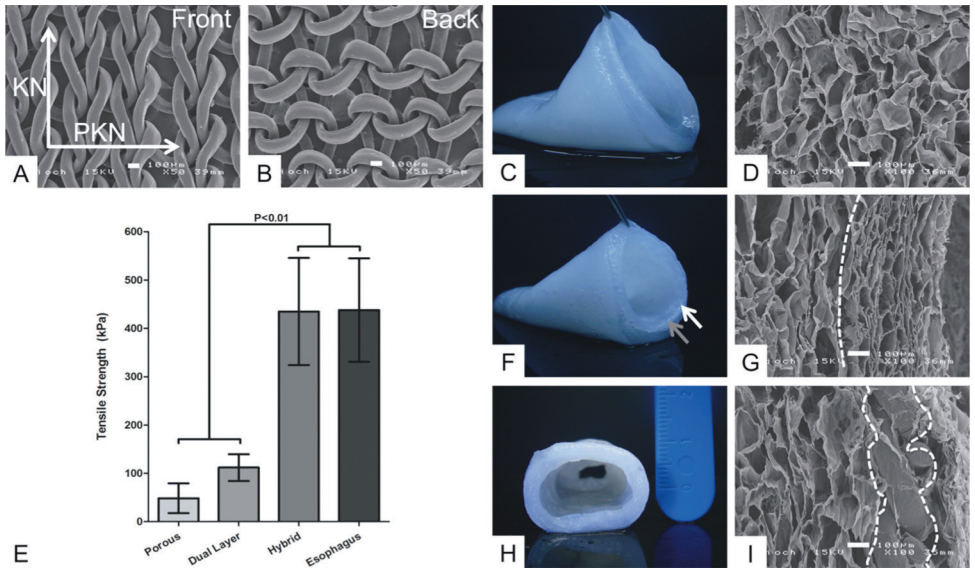

FIGURE 2: SCAFFOLD CHARACTERISTICS.

A) and **B)** Scanning electron microscopy (SEM) images of the polymer knitting structure of the front and back. A difference in structure can be seen where the knitting direction (KN) displays elastic behavior, whereas the perpendicular direction (PKN) is more stiff and rigid. Macroscopic appearance and SEM images of the three types of scaffolds. **C)** and **D)** single-layered porous scaffold. **F)** and **G)** dual-layered semi porous scaffold, where the white dotted line represents the boundary between porous and the compressed, less porous layer. **H)** and **I)** Hybrid scaffold, where the white dotted line indicates the location of the polymer knitting. **E)** Tensile strength was determined for all three scaffolds and compared with an adult porcine esophagus, error bars represent standard deviation; all white scale bars represent 100 µm.

3.2 SCAFFOLD CHARACTERIZATION

SEM analysis of all scaffolds displayed highly porous material with pore sizes ranging from 50–120 µm (Figure 2D, G, I). Both layers in the dual layer scaffold were attached in which the boundary is indicated by the white dotted line (Figure 2G). The polymer knitting was fully incorporated in the hybrid scaffolds, and an interaction was observed between collagen and the knitting (Figure 2I). The three different scaffolds (single-layered, dual-layered, and hybrid scaffolds) showed a decrease in free amine groups of 47%±5%, 61%±1%, and 39%±1% (n=3), respectively, in comparison to non-crosslinked samples (data not shown). Furthermore, the scaffold thickness and ultimate tensile strength (UTS) was measured (Figure 2E). The tensile strength data were expressed in Newton (N) at break, and these were subsequently corrected for scaffold thickness, resulting in Pascal (Pa). Thickness and strength values for the single-layer, dual-layer, and hybrid scaffolds were 3.7±0.6 mm and 48±30 kPa; 3.4±0.3 mm and 112±28 kPa; and 3.3±0.1 mm and 435±110 kPa, respectively. As a reference, tested native esophagus showed a rupture strength of 438±106 kPa (3.5±0.1 mm). The hybrid scaffold was significantly stronger than the single- and dual-layer scaffold ($p<0.01$). Moreover, the hybrid scaffold was equally strong as the native porcine esophagus. The polymer knitting incorporated in the hybrid scaffold displayed anisotropic mechanical behavior. This was quantified as 1559±316 kPa in the KN, and 2800±305 kPa PKN. The absolute strength (expressed in Newton [N]) of the polymer knitting alone and the hybrid scaffold were comparable. However, data conversion to tensile strength (expressed in Pascal [$N.m^2$]) consigns a higher tensile strength to the polymer knitting, as the hybrid scaffold's wall is thicker in comparison to the polymer knitting's cross-section.

3.3 VASCULARIZATION BY OMENTUM WRAPPING

Both single- and dual-layered scaffolds required placement of stents in their lumen to retain patency. Hybrid scaffolds (indicated by white arrows) did not require stent placement, but stents were placed for standardization (Figure 3). All three sheep underwent the procedure without surgical complications. One animal was sacrificed after 11 days due to wound infection, which was in accordance to animal

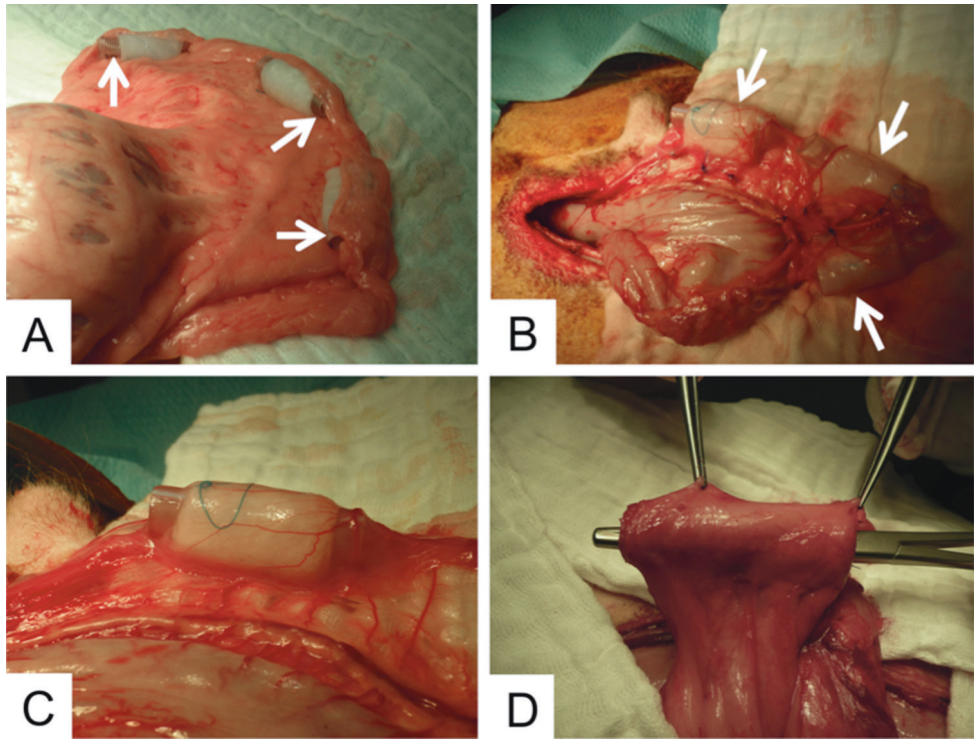

FIGURE 3: PHOTOGRAPHS OF SCAFFOLD IMPLANTATION AND OMENTUM WRAPPING IN SHEEP.

A) The exteriorized omentum was wrapped around the three differently constructed collagen-based scaffolds, all of which were stented by silicone tubing (white arrows). **B)** and **C)** Sutures were placed to prevent the omentum from unwrapping. **D)** Enwrapped scaffolding was exteriorized, examined for patency, and removed after 6 weeks *in vivo* for immunohistological evaluation.

welfare guidelines. The two other animals were sacrificed 6 weeks post-surgery as scheduled. Attachment of the omentum to the tubular scaffolds was observed as firm in all cases. Separation of the implanted scaffolds from the omentum was not possible. The omentum covering the tubular scaffolds displayed the same macroscopical vascularization as the omentum.

In all scaffolds, cells had infiltrated the scaffold and matrix material was deposited as indicated by 4′,6-diamidino-2-phenylindole (DAPI), HE and MT staining as exemplified for the hybrid scaffold (Figure 4A–D). Cells were present in both the collagenous regions and the surrounding PCL layer (Figure 4A). Omental attachment was observed in all cases with a clear boundary between the fatty omentum and the collagen scaffold (Figure 4B). Vascularization was substantial, and mature blood vessel-like structures were found throughout all scaffolds (Figure 4C, D). These structures were positive for type IV collagen (Figure 4E and F). Calcification was absent throughout the collagenous areas and around the PCL knitting, as indicated by the von Kossa staining (Figure 4G). In addition, throughout scaffolds, cells were exposed to normoxic conditions as indicated by the negative for HIF-1α, which is a marker for hypoxic cells. However, some slight staining for HIF-1α was observed in direct vicinity of the polymeric structures in the hybrid scaffolds (Figure 4H).

4. DISCUSSION

RM for the esophagus holds promise, especially in the setting of pediatric patients with congenital birth defects and adult patients with malignancies or recurrent strictures. Currently, autologous donor tissue is generally used to replace the diseased of damaged segments. The procurement of autologous tissue, especially from the lower gastrointestinal tract, remains associated with the development of serious complications. Here, we studied three tubular, porous collagen scaffolds as an alternative for autologous tissues. Collagen scaffolds were successfully reinforced by the addition of a compressed collagen layer or synthetic polymer knitting. However, only the hybrid scaffolds were superior with regard to UTS and approached the strength of a mature porcine esophagus. As previously mentioned,

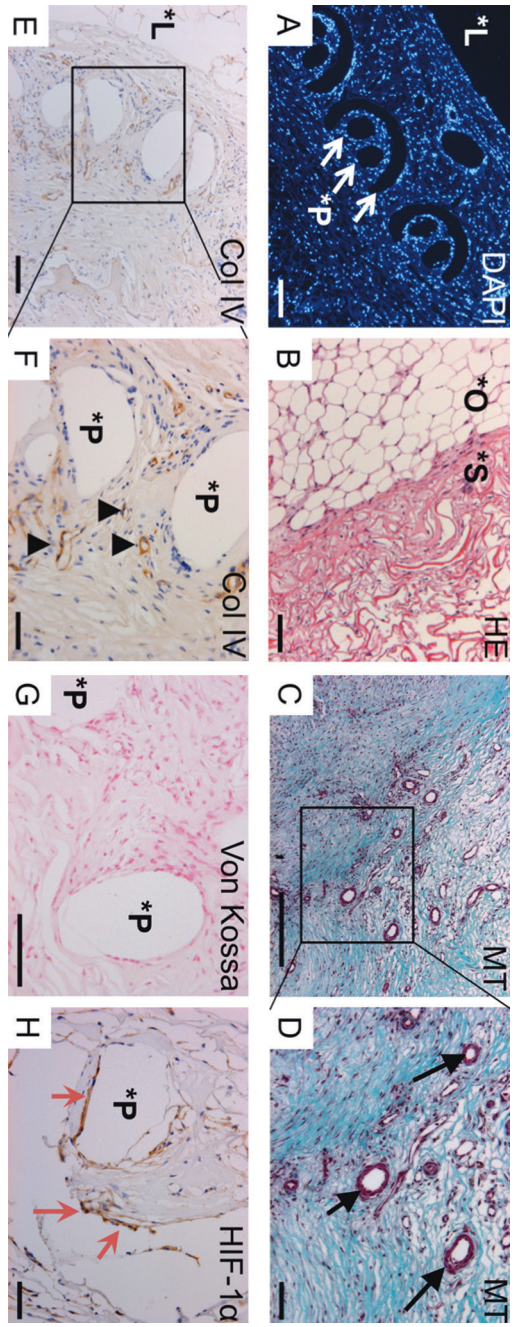

FIGURE 4: HISTOLOGICAL EVALUATION OF THE HYBRID COLLAGEN CONSTRUCTS.

A) Cell infiltration demonstrated by DAPI staining, arrows show polymer imprints, scale bar represents 200µm. **B)** HE staining showing omental integration of the scaffold, scale bar represents 50µm. **C)** MT staining showing vascularization of the collagen scaffold, scale bar represents 50µm. **D)** Close-up of C showing blood vessels (black arrows) 200×, scale bar represents 200µm. **E)** Type IV collagen-type staining indicates blood vessels near polymer knitting, scale bar represents 200µm. **F)** Close-up of E showing blood vessels (indicated by black arrow heads) 200×, scale bar represents 50µm. **G)** Von Kossa staining indicating absence of calcification, 400×, scale bar represents 50µm. **H)** Local hypoxic conditions indicated by positive HIF-1α staining, only in the immediate vicinity of the polymers as shown by red arrows, 200×, scale bar represents 50µm. ***L**, lumen; ***O**, omentum; ***S**, scaffold; ***P**, polymer knitting. Col IV, Type IV collagen; DAPI, 4'6-diamidino-2-phenylindole; HE, hematoxylin and eosin; HIF-1α, hypoxia-inducible factor 1-alpha; MT, Masson trichrome.

the anisotropic mechanical characteristics of the polymer knitting were incorporated into the scaffold such that peristaltic movements may be facilitated. In addition, we show that *in vivo* omentum wrapping leads to vascularization of the scaffolds, with blood vessels of a different diameter. Differences in degree of vascularization were not observed between the different scaffold types. This indicates the robustness of omentum wrapping and appears to be a clinically feasible technique for large scaffolds. Moreover, it may prove to be an essential step before surgical intervention.[42, 43] A tubular-shaped scaffold for esophageal reconstruction has previously been created by the seeding of primary esophageal cells on flat bovine collagen sheets sutured into a tubular shape.[44, 45] Circumvention of the error-prone additional tubularization steps may lead to a more controllable surgical procedure. Moreover, in the case of pediatric applications, the scaffolds should encompass the patient's growth rate with less chance of leakage and tearing at the suture site due to the pre-tubularized shape. We have previously shown that molecularly defined scaffolds can be prepared from highly purified insoluble type I collagen fibrils.[36] In combination with lyophilization techniques, its potential as a biomaterial has been demonstrated.[32, 46] Moreover, the specific production process allows for interchangeable scaffold dimensions; for example, tube diameter, wall thickness, and/or length by changing the molding and casting tools, thus allowing patient-specific scaffolds.[35] Lyophilization of the casted collagen suspensions leads to a defined and controllable porous microenvironment, which allows cells from surrounding tissue to infiltrate, facilitating formation of new tissues.[47, 48]

Although ex vivo pre-vascularization of large scaffolds has been investigated (mainly aimed at prevention of fibrosis), we opted for *in vivo* omentum wrapping, as it is a clinically feasible technique for large scaffolds and may prove to be an essential step before surgical intervention.[42, 43] Inflammatory response was generally very mild. This can be explained by the high purity of the collagen and by the exposition of the scaffold to omental tissue only and not to an open wound. Apart from the vascularization of all three scaffolds, blood vessels were also found near the PCL polymer knitting, indicating that it does not hinder the vascularization process. The normoxic conditions observed throughout the scaffolds (except for areas in the direct vicinity of the PCL knitting) indicate that the vascularization is probably sufficient to support the current number of cells in the scaffold.

Since this study was meant as a proof of principle, the proposed scaffolds may represent a platform for further optimization and future *in vivo* studies. For instance, growth factors may be incorporated to stimulate processes as cell growth and differentiation, angiogenesis, maturation of blood vessels, and ingrowth of muscle cells.[46, 49] Other biologicals such as, for example, fibrin, elastin, and laminin, may be added to further optimize and fine-tune the scaffold. In the future, next to the tubular collagenous construct, the knittings also will have been produced in a fully seamless tubular structure. In conclusion, different large-diameter tubular scaffolds were constructed from molecularly defined materials, which showed adequate biocompatibility and vascularization after omentum wrapping. The collagen-PCL scaffold was superior in strength, and it makes a good candidate scaffold for further fine-tuning for future esophageal repair or replacement.

ACKNOWLEDGEMENTS

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CHAPTER 04

TISSUE ENGINEERED TUBULAR CONSTRUCT:

URINARY DIVERSION IN A PRE-CLINICAL PORCINE MODEL

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ABSTRACT

The ileal conduit has been considered the "golden standard" urinary diversion for bladder cancer patients and paediatric patients. Complications are mainly related to the use of gastro-intestinal tissue (GIT). Tissue engineering may be the technical platform to develop alternatives for GIT. This study aims to develop a collagen-polymer conduit, and evaluate its applicability for urinary diversion in pigs. Large tubular constructs (l=12 cm, Ø=15 mm) were prepared from bovine type I collagen and a synthetic polymer mesh (Vypro® II mesh, Ethicon, Inc., Somerville, NJ, USA). Characterized tubes were sterilized, seeded with and without primary porcine bladder urothelial cells (UC), and implanted as an incontinent urostomy, using the right ureter, in ten female Landrace pigs. After one month the newly formed tissue structure was functionally (loopogram) and microscopically (immunohistochemistry) evaluated. Survival rate was 80% (one related and one unrelated death). At one month, the collagen was resorbed and a retroperitoneal tunnel was formed which could withstand water pressure of 40 cmH_2O. In five cases the tunnel functioned as a urostomy. Histological analyses revealed a moderate immune response, neo-vascularisation, and the presence of urothelial cells in the lumen of the construct. The polymer mesh provoked fibroblast deposition and tissue contraction. No major differences were observed between cellular and acellular constructs. After implanting the tubular constructs, a retroperitoneal tunnel was formed which functioned, in most cases, as a urinary conduit. Improved large tubular scaffolds may generate alternatives for the use of GIT in urinary diversion.

1. INTRODUCTION

The uretero-ileo-cutaneous conduit (or urostomy) has been the primary technique for incontinent urinary diversion for over 50 years.[1] Despite the complication risks, ~33% of the urologists opt for this technique.[2] Complications range between 20-56% within the first weeks after surgery, and can increase to 28-81% after one month.[3,4] Early complications are usually related to the bowel resection and anastomosis, while long-term complications are more stoma related.[5] Bowel related complications may include anastomotic leakage, enteric fistula, bowel obstruction, and prolonged ileus. Therefore, it is desirable to prevent the use of gastro intestinal tissue (GIT), and to create a urinary conduit from an artificial construct through which urine can be diverted. The pre-operative care will be less intensive and surgery time will be reduced (e.g. no special diets or medication to clean the bowel). Bowel complications will be reduced, and hospitalisation will be shortened.[6] Although many (bio)materials have been developed, the number of studies which have investigated artificial urinary conduits is minimal. In 2007, Drewa *et al*. has successfully demonstrated urinary diversion in rats using an artificial cell seeded conduit.[7] Other groups have been developed artificial tubes, which are either epithelialized tubes prepared from minced autologous combined with fibrin, or tubular degradable biopolymeric scaffolds seeded with epithelial and smooth muscle cells.[8-10] Although the outcome is promising, the *in vivo* performance for urinary diversion has to be demonstrated. Utilizing this knowledge, we developed a new large diameter construct and investigated its applicability and function for an incontinent urinary diversion in a preclinical pig model. This to confirm if a tissue engineered conduit may be useful to replace the bowel tissue.

2. MATERIALS AND METHODS

2.1 PREPARATION OF LARGE TUBULAR COLLAGEN-POLYMER SCAFFOLDS

Type I collagen was purified at Symatese biomateriaux, Chaponost, France, according to a earlier described protocol.[11] Large tubular constructs (l=12 cm, Ø=15 mm) were prepared by combining homogenized collagen (0.7% w/v) with and without a tubularized Vypro® II mesh (Ethicon, Inc., Somerville, NJ, USA) in a cylindrical mould, followed by subsequent freezing, and freeze-drying techniques.[12] Dried materials were crosslinked using carbodiimide crosslinking, freeze-dried again and sterilized using 25 kGy gamma irradiation (Isotron, Ede, The Netherlands).[13]

2.2 SCAFFOLD CHARACTERIZATION

The biochemical composition, mechanical properties and morphology of the scaffolds were characterized. The degree of collagen crosslinking was measured using 2,4,6-trinitrobenzene sulfonic acid (TNBS).[13] Ultimate tensile strength (UTS) was determined on 8x30 mm pre-wetted strips at an elongation speed of 2.9 mm/min (n=3) using the Bose BioDynamic bioreactor (Bose ElectroForce, Eden-Prainie, MN, USA). Scanning electron microscopy (SEM) was used to analyse the ultra-structure of the tubular scaffolds.[14]

2.3 *IN VIVO* STUDY

All procedures were performed according to the Institute of Laboratory Animal Research (ILAR) guide for laboratory animals.[15] This study was approved by the Animal Ethics Committee of the Radboud University Medical Center. Ten female Landrace pigs (~50 kg) were used in the present study. They were housed individually and had a restricted diet and water ad libitum.

2.3.1 UROTHELIAL CELL ISOLATION AND SEEDING IN TUBULAR CONSTRUCTS

Bladder tissue (~4 cm^2) was harvested from the pigs under general anaesthesia through a midline incision in the lower abdomen. The bladder biopsy was immersed in transport medium (Hanks' Balanced Salts (HBSS) with Ca^{2+} and Mg^{2+}, 10 mM HEPES, 0.1% aprotinin, 1% penicillin/streptomycin). For enzymatic treatment, the biopsy tissue was placed in stripping medium (HBSS without Ca^{2+} and Mg^{2+}, 10 mM HEPES, 0.1% aprotinin, 1% penicillin/streptomycin (P/S), 2.4 U/mL dispase II) o/n at 4°C [16]. The urothelium was removed using forceps and digested in collagenase IV solution (HBSS with Ca^{2+} and Mg^{2+}, 10 mM HEPES, 100 U/mL collagenase IV) for 20 min at 37°C. The urothelial cells (UC) were suspended and collected in keratinocyte serum free medium (K-SFM (Invitrogen, Paisley, UK) with 0.5 ng/mL EGF, 5 ng/mL bovine pituitary extract, 30 ng/mL cholera toxin and 100 U Penicillin/ml and 100 µg Streptomycin/ml PBS), centrifuged, resuspended and cultured on a mouse fibroblast feeder layer (STO) in T75 Primaria flasks (BD, Franklin Lakes, NJ, USA). After 4 weeks, both ends of the tube were surgically closed using 2.0 Vicryl sutures and UC were seeded by injecting 10x106 in the lumen of the construct. After o/n attachment of the cells, the constructs were opened and cultured for 6 days. Constructs for the acellular group were kept in K-SFM until implantation.

2.3.2 IMPLANTATION OF TUBULAR SCAFFOLD IN UROSTOMY MODEL

The animals were divided into two groups, one group (n=4) received an acellular construct while the other group (n=6) received a seeded construct (Figure 1). A median incision was performed and via an extra-peritoneal route the retroperitoneal cavity was entered. The right ureter was located, mobilized, and transected. After gaining sufficient length (~20 cm), the ureter was spatulated, and a tension free end-to-side anastomosis was performed using 5-0 Monocryl (Ethicon) running sutures. The distal part of the ureter was closed. A flank incision was made (through skin, fascia and muscle) on the right lateral side before the hind leg, through which the tubular construct was led. A ureteral catheter (8 Fr, Vycon, Ecouen, France) was

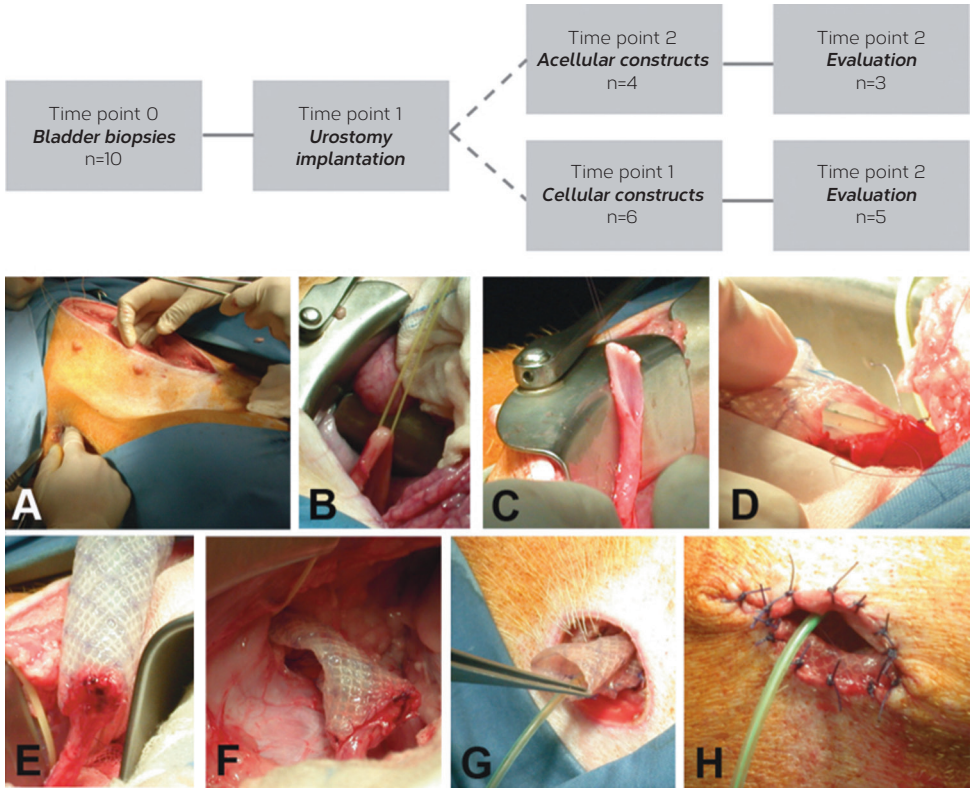

FIGURE 1: OVERVIEW OF THE UROSTOMY CONSTRUCTION IN A PIG MODEL.

After one month (time point 1) the animals were divided into two groups, one group (n=4) received an acellular construct while the other group (n=6) received a construct seeded with autologous urothelial cells. **A)** Midline incision just below the umbilicus was performed, **B)** localisation of the right kidney and right ureter, **C)** dissection and spatulation of the right ureter. **D)** and **E)** Anastomosing the scaffold end-to-side to the proximal ureter using 5-0 Monocryl running sutures. **F)** Passage of construct through the abdominal wall. **G)** and **H)** Attachment of the construct to the fascia and skin. A straight catheter (8 Fr) was left in the ureter for two weeks.

inserted and fixed using a 4.0 Monocryl (Ethicon) at the distal part of the construct. The construct was fixed to the fascia using 2.0 Vicryl sutures and to the skin using 3.0 Vicryl sutures (both Ethicon). The laparotomy was closed using 0 Vicryl sutures for the fascia, 2.0 Vicryl running sutures for the subcutaneous fat and 1 CT Vicryl for the skin (all Ethicon). The catheter was removed after 21 days.

2.4 CHARACTERIZATION OF UROSTOMY

One month after the urostomy implantation the animals were evaluated radiologically and histologically. A loopogram was performed by instilling 1:1 diluted Xenetix 300 iodinated contrast fluid (Codali Guerbet, Brussels, Belgium) in the construct via a catheter to a pressure of 40 cmH_2O. All images were collected with a Philips BV-25 C-arm image intensifier (Philips, Eindhoven, The Netherlands). Thereafter, the animals were sacrificed using an overdose of intravenous barbiturate. The urostomy, ureter and kidney were macroscopically inspected and fixed in 4% (v/v) formaldehyde (in PBS) for further histological evaluation.

2.5 IMMUNOHISTOCHEMISTRY

Paraffin embedded material was cut and stained with Haemotoxylin-eosin (H&E).[17] Additionally, immunohistochemistry (IHC) for pancytokeratin, smoothelin, vimentin, desmin and alpha smooth muscle actin was performed. The sections were deparaffinized and blocked in 3% (v/v) H_2O_2 in PBS. Antigen retrieval was performed for pancytokeratin and smoothelin using microwave treatment. All sections were incubated with 5% (w/v) horse serum for 30 min. Sections were incubated with primary antibodies for 1h. A subsequent reaction was performed using ABC kit (Vector Lab, Burlingame, CA, USA) and developed utilizing PowerDAB (Diaminiobenzidine, ImmunoLogic, Duiven, The Netherlands). Sections were counterstained with Haematoxylin (Boom BV, Meppel, The Netherlands).

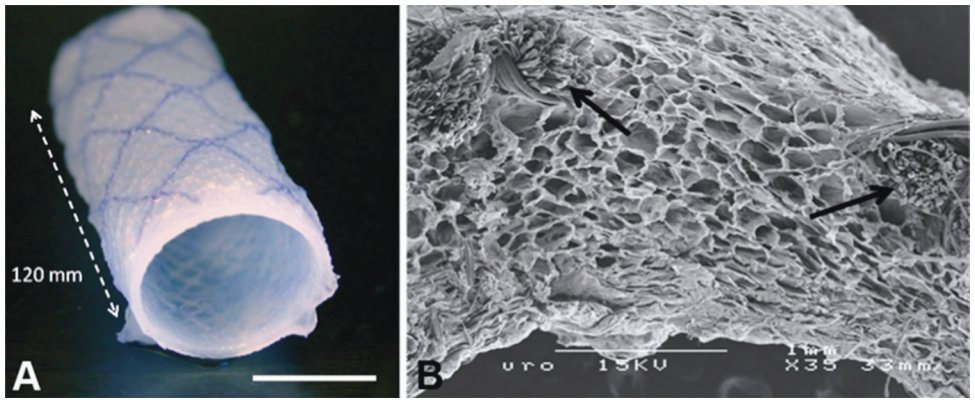

FIGURE 2: MACROSCOPIC AND MICROSCOPIC APPEARANCE OF THE TUBULAR CONSTRUCT.

A) Macroscopic overview (l = 12 cm, Ø = 15 mm). Please note the inclusion of Vypro® II mesh (blue) **B)** scanning electron image of the cross-section of the scaffold indicating a highly porous network. Black arrows mark the fibres of the polymer mesh. Please note the intimate contact between the polymer mesh and collagen. Polymer fibres were not observed in the lumen of the scaffold.

3. RESULTS

3.1 SCAFFOLD CHARACTERIZATION

A macroscopic overview of the collagen-polymer large tubular constructs is shown in figure 2. The tubular constructs were highly porous (pore size ranging 100–150 μm; Figure 2B) and the polymer mesh was fully incorporated. The collagen was successfully crosslinked as evidenced by a decrease of 37% in free amine groups. The tensile strength of the hybrid constructs was 5-fold higher (1.25 ± 0.19 N/mm) than a similar construct prepared from collagen only (0.25 ± 0.04 N/mm).

3.2 CELL SEEDING AND CULTURE

After one week of static culture (Figure 3A1), an almost confluent layer of UC was observed lining the lumen of the construct (Figure 3A, B). IHC staining showed that seeded cells were positive for pancytokeratin (Figure 3C), confirming that only urothelial cells were seeded on the scaffolds.

3.3 *IN VIVO* EVALUATION

Survival rate after the urostomy implantation was 80% (8/10). One animal showed severe weight loss and lethargy and was therefore sacrificed according to regulatory guidelines (unrelated death). Stoma site stenosis occurred in all animals. In one animal a complete closure of the conduit was observed, and this animal was also sacrificed before the predetermined time point (related death). To prevent stomal stenosis, wound drains (Microtek Medical) were positioned inside the urostomy and fixed to the distal part of the stoma and to the skin, and were left in place till the end of the experiment. In all other animals no complete obstruction was observed. In the eight animals that survived the follow-up period, a retroperitoneal tunnel was formed through which urine was diverted (Figure 4A). Loopograms showed stenosis at the ureter anastomosis in three animals, from which two had leakage. In the remaining five animals the urostomy could withstand water infusion at a pressure of 40 cmH_2O. The mean length and outer diameter of the conduits was 10.2 ± 1.5 cm and 2.7 ± 0.4

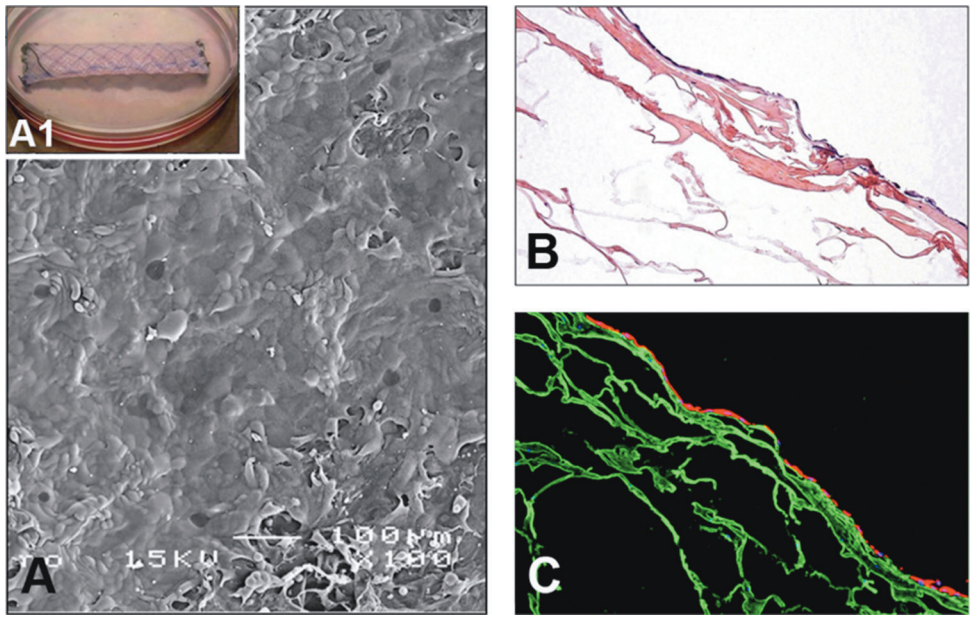

FIGURE 3: MICROSCOPIC ANALYSIS OF PRIMARY UROTHELIAL CELLS AFTER SEEDING.

A) Scanning electron microscopy demonstrating a nearly confluent layer of urothelial cells (UC) covering the lumen. **B)** Hematoxylin-eosin stained cross-section showing cellular lining **C)** pan-cytokeratin stained positive for cellular lining inside the construct, indicating that the seeded cells are UC. **A1)** Macroscopic picture of tubular scaffold in culture.

cm respectively (Figure 4C and E). The lumen of these structures had a star shaped appearance (Figure 4D and 5D). In four cases, polymer mesh was found in the lumen of the conduits, and was neither degraded nor incorporated in the tissue. In all pigs the right upper urinary system was affected, showing a hydro-ureter and hydro-ureteronephrosis (Figure 4B).

3.4 HISTOLOGY

Differences between the acellular and cellular group were not observed. Nearly all collagen scaffold material was replaced by extracellular matrix (ECM) (Figure 5). In some of the animals (6 out of 8) parts of the lumen contained pancytokeratin positive cells confirming a neo-epithelial development (Figure 5A and F). Vimentin staining was positive (Figure 5B), whilst most of the subluminal tissue was negative for desmin, smoothelin (data not shown) and smooth muscle actin (except for the vasculature, see Figure 5C and G), indicating the presence of fibroblasts and absence of smooth muscle cells. A moderate immune response was observed including some multinucleated giant cells (Figure 5E). Neo-vascularisation was seen throughout the entire urostomy (Figure 5G). The regeneration at the site of ureteric anastomosis was more developed with a continuous urothelial lining and smooth muscle cell ingrowth (Figure 6).

4. DISCUSSION

The most widely used solution for urinary diversion is still the ileal conduit. A large tubular construct that can function as an artificial conduit may simplify the surgical procedure and could reduce GIT related complications.[6] In this study, we prepared a large tubular collagen-polymer scaffold, with the length of the bowel segments currently used for urostomy, and tested its performance as an artificial conduit in a preclinical pig model.[18] Successful implantation of the construct was achieved. A watertight anastomosis between the ureter and the construct could be made. One month after implantation the collagen was resorbed and a highly vascularized retroperitoneal tunnel was formed through which urine diverted.

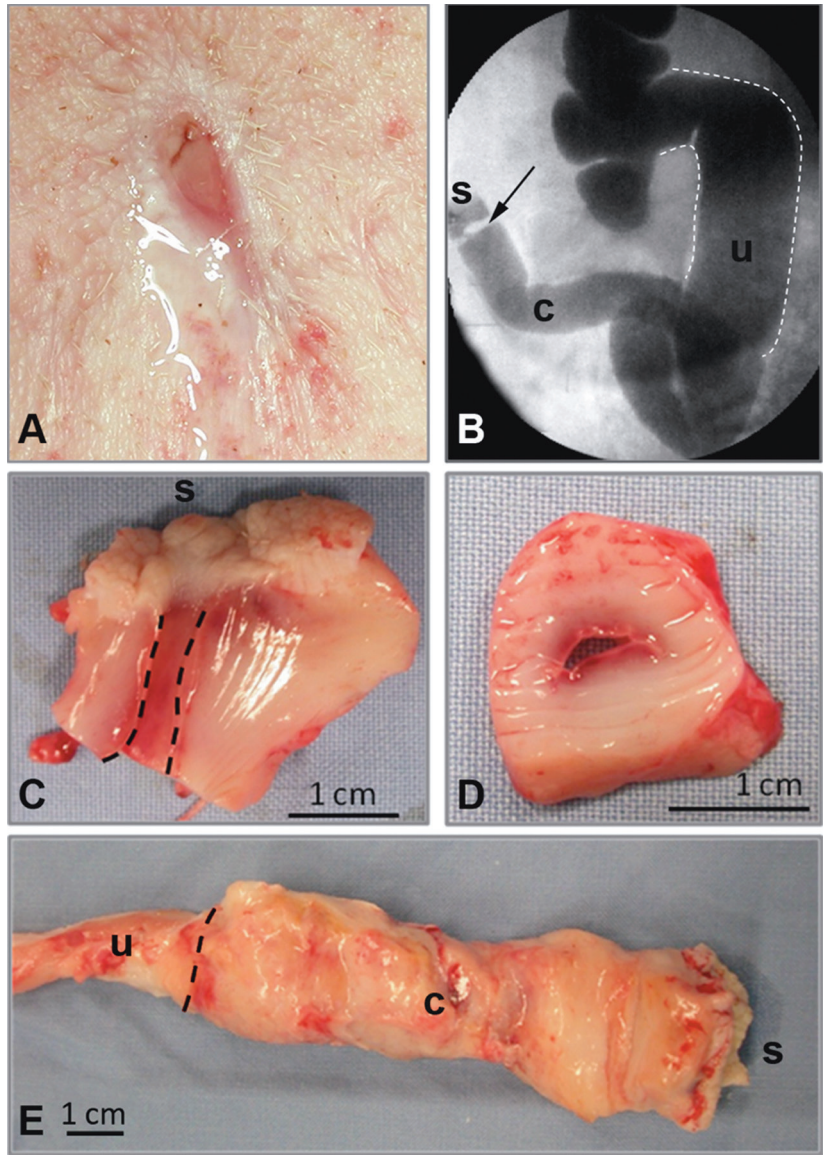

FIGURE 4: MACROSCOPIC OVERVIEW AND LOOPOGRAM OF THE UROSTOMY 1 MONTH POST-SURGERY.

A) Opening at skin level with urine. **B)** Loopogram demonstrating a stenosis at skin level (black arrow), a severely dilated ureter (white dotted line) and a dilated renal pelvis. **C)** Longitudinal cross-section of conduit at skin side. **D)** Cross-section of conduit. **E)** Complete resected urinary conduit. u = ureter, s = skin site, c = conduit. Bar represents 1 cm.

Although 80% of the animals survived the experiment, post-mortem examination showed that all animals had a hydronephrotic kidney and a hydro-ureter. These findings were probably caused by stenosis at the skin side of the stoma.

Several factors, including the use of 3D biomaterials are important when developing such artificial conduits.[8, 19, 20] We incorporated a polymer mesh to reinforce the fragile, easily collapsible collagen, which resulted in reinforcement of the construct without affecting the cytocompatibility. An almost confluent layer of urothelial cells was observed on the luminal side of the scaffold within one week of culture. This indicates that the construct is adequate for stable attachment, proliferation and survival of other primary cells, as seen in a previous study.[21] After one month *in vivo*, urothelial cells were found in 6 out of 8 conduits (in both seeded and unseeded groups). Epithelial outgrowth and SMC ingrowth was observed, particularly at the end of the construct with the ureteric anastomosis. This indicates that the generation of a successful urostomy may not be dependent on urothelial pre-seeding.

Vypro® II mesh was not incorporated in the tissue, indicating that it may not be biocompatible, and should be replaced by a mesh produced from another material. The ideal polymer mesh for this purpose should be biocompatible, pre-tubularized, EMA and FDA approved. Unfortunately, such a polymer mesh is currently unavailable. We hypothesized that the polymer mesh should support the mechanical load until sufficient tissue regeneration has occurred. Vicryl and Dexon meshes were not used because they degrade within weeks and lose adequate support. However, a recent study reported that Vicryl mesh induces a less severe inflammatory reaction than Vypro® II mesh when implanted retroperitoneally in a pigs.[22] In view of the rapid formation of the retroperitoneal tunnel in our study, it is foreseeable that the rapidly resorbable meshes Vicryl and Dexon could be used, and new tubular biodegradable polymer structures are necessary to meet the requirements.

Although the implanted tubular constructs functioned as a urinary conduit, post-mortem examination showed the presence of a hydro-ureter and a hydronephrotic kidney on the side of the urostomy in all animals. Most likely, this was caused by the stomal stenosis, which may be the consequence of the use of the Vypro® II mesh. This is in accordance with other studies showing that this mesh can provoke fibroblast deposition and skin contraction, and is poorly biocompatible.[22] It is known that the addition of cells decreases fibroblast deposition and therefore reduces myofibroblast

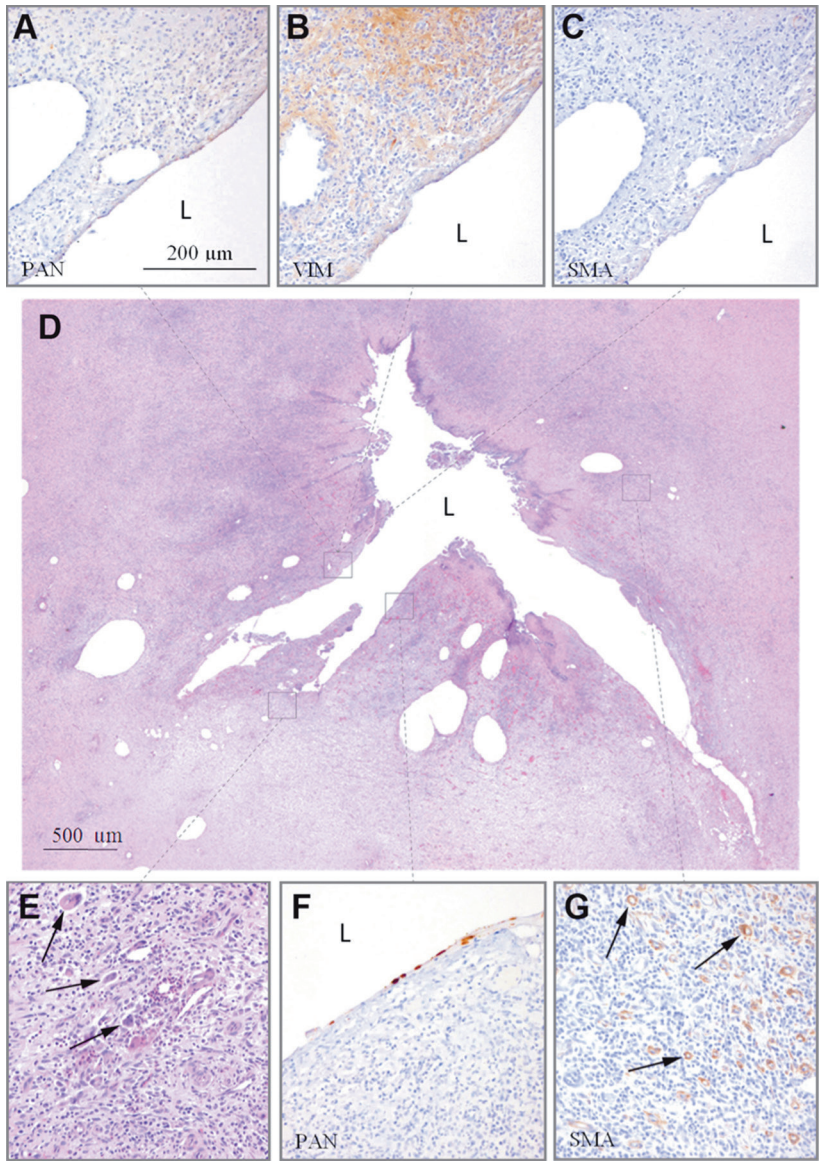

FIGURE 5: IMMUNOHISTOGRAMS OF UROSTOMY TISSUE.

A) and **F)** Inhomogeneous lining of the urostomy with pan-cytokeratin positive cells (PAN). **B)** Heterogeneously distributed vimentin-positive cells (VIM). **C)** Absence of smooth muscle actin-positive cells (SMA). **D)** Overview photo of conduit lumen and surrounding tissue. **E)** Presence of granulocytes and some multi-nucleated giant cells (arrows). **G)** Presence of multiple capillaries throughout the entire urostomy (arrows). Bars represent 200 and 500 µm. L = lumen of conduit.

related contraction.[9, 23, 24] However, we did not observe a positive effect of the applied UC, and we assume that the incompatible response of the mesh diminished this effect. On the other hand, more cell types may be needed in order to generate a urostomy resembling an ileal-like conduit. Since the smooth muscle compartment of the bowel segment has contractile activity, the introduction of contractile smooth muscle cells in the wall of the tissue engineered construct may be important to generate peristaltic movement and prevent hydronephrosis. In this respect, careful consideration should be taken to determine whether a cellular construct out-weighs the disadvantages, i.e. increased costs and decreased clinical applicability.

Other unavoidable features which may also contribute to the hydronephrosis are the quadripedal stance, rapid growth and high intra-abdominal pressures of the animal models.[10, 25] A propensity for stomal stenosis was described in three other studies.[25-27] All studies mentioned that the stomal complications were caused by the intrinsic skin healing properties of pigs, similar to our observation. Despite these limitations, there are not many alternatives to the use of the pig model. An important factor is the relevance to the human situation since the size and abdominal anatomy are most analogous.[28, 29] Changing the animal model might decrease stomal stenosis and obstruction, but may diminish the clinical relevance. In addition, hydronephrotic kidneys and stenosis were also seen in a rat model with urinary diversion.[7] Finally, we did not evert the construct in contrast to the customary technique in humans, where the gastro-intestinal segment is everted to diminish leakage between the stoma and the collection bag. Everting the ileal conduit in a porcine model prevented stenosis of the conduit at skin level.[3] Accordingly, eversion of the tubular construct may be needed to alleviate the stenosis observed and facilitate successful stoma bag fitting.

5. CONCLUSIONS

Our study showed that large diameter tubular constructs with defined mechanical and structural characteristics were produced, which, when used as a urinary conduit, generated a retroperitoneal tunnel through which urine was diverted. Since we found no significant differences between the

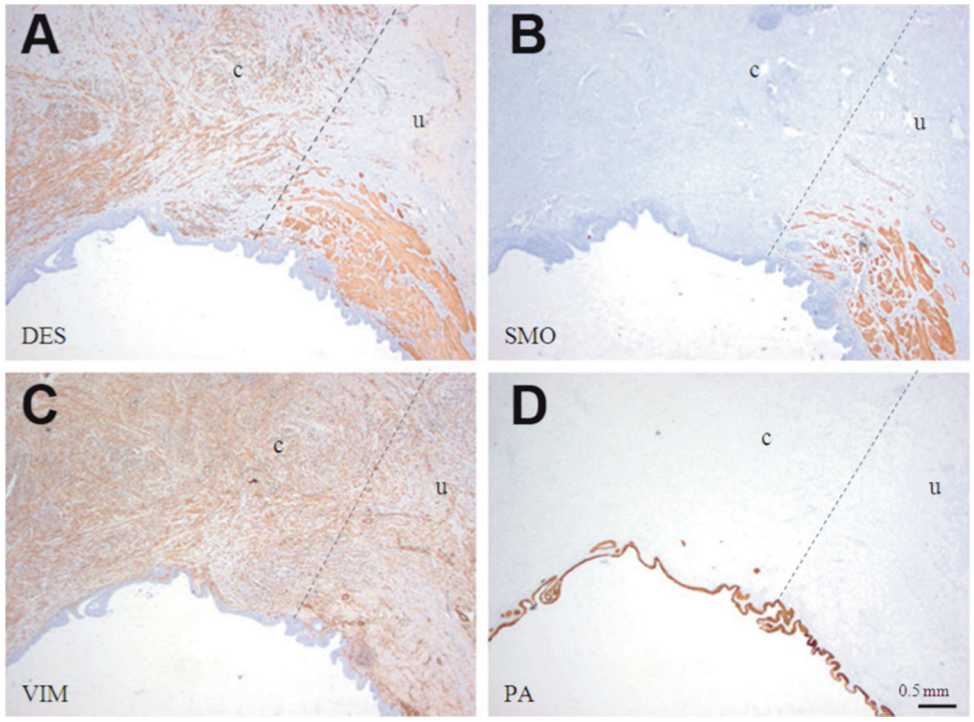

FIGURE 6: IMMUNOHISTOCHEMISTRY OF THE URETER ANASTOMOSIS *(INDICATED WITH A DOTTED GREY LINE).*

A) Ingrowth of smooth muscle cells was shown by a positive signal for desmin (DES). **B)** The remodeled construct was negative for smoothelin (SMO) indicating a proliferative (early) stage of smooth muscle development. **C)** Tissue was positive for vimentin (VIM). **D)** Continuous lining of ureter and construct with pancytokeratin-positive cells (PAN). u = ureter, c = conduit. Bar represents 0.5 mm.

groups, we conclude that pre-seeding UC may not provide any advantage by virtue of the development of an urothelium. Although the current design needs to be improved, the tissue engineered tubular constructs may be developed into an alternative for GIT tissue and could represent a potential readily available product for urinary diversion surgery.

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CHAPTER 05

COLLAGEN FIBER ALIGNMENT:

DIRECTING COLLAGEN FIBERS USING COUNTER-ROTATING CONE EXTRUSION

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ABSTRACT

The bio-inspired engineering of tissue equivalents should take into account anisotropic morphology and the mechanical properties of the extracellular matrix. This especially applies to collagen fibrils, which have various, but highly defined, orientations throughout tissues and organs. There are several methods available to control the alignment of soluble collagen monomers, but the options to direct native insoluble collagen fibers are limited. Here we apply a controlled counter-rotating cone extrusion technology to engineer tubular collagen constructs with defined anisotropy. Driven by diverging inner and outer cone rotation speeds, collagen fibrils from bovine skin were extruded and precipitated onto mandrels as tubes with oriented fibers and bundles, as examined by second harmonic generation microscopy and quantitative image analysis. A clear correlation was found whereby the direction and extent of collagen fiber alignment during extrusion were a function of the shear forces caused by a combination of the cone rotation and flow direction. A gradual change in the fiber direction, spanning +50 to −40°, was observed throughout the sections of the sample, with an average decrease ranging from 2.3 to 2.6° every 10 μm. By varying the cone speeds, the collagen constructs showed differences in elasticity and toughness, spanning 900–2000 kPa and 19–35 mJ, respectively. Rotational extrusion presents an enabling technology to create and control the (an)isotropic architecture of collagen constructs for application in tissue engineering and Regenerative Medicine.

1. INTRODUCTION

1.1 HETEROGENEITY OF THE EXTRACELLULAR MATRIX *IN VIVO*

Biomaterials that can mimic the target extracellular matrix (ECM) morphology and mechanical properties are crucial for the success of tissue engineering and Regenerative Medicine strategies.[1] Tissue structure is generally heterogeneous, and frequently displays the anisotropic organization of the cells and the surrounding ECM. Fibrillar collagen, the main component of the ECM, which has a major impact on the mechanical properties of tissues, shows remarkable variability of fibrillar organization, density and alignment.[2, 3] The architecture of collagen fibers of e.g. the skin (basket-weave orientation) and tendon (parallel oriented) are highly defined, as is the fiber orientation in organs such as bladder, artery, cartilage and bone.[4-9] Controlling the collagen fiber organization upon scaffold construction is critical for achieving a controlled impact on matrix-influenced complex cell functions, including proliferation, differentiation and migration.[10-13] The importance of ECM geometry on cell function is illustrated by the orientation of cell-derived ECM deposition, which follows the orientation of the pre-existing template.[14]

1.2 BIO-INSPIRED GENERATION OF COLLAGEN FILMS

Few approaches allow the generation of anisotropic fibrillar collagen constructs from native collagen fibers and/or fibrils due to the insoluble nature of collagen. Scaffolds prepared from native collagen fibrils rather than from soluble collagen molecules may be preferential since collagen fibrils harboring native crosslinks and molecular alignment (quarter staggered array) are the structural components that cells encounter in the body. Techniques based on weaving, casting and/or freezing are the only enabling technologies that can produce constructs consisting of insoluble collagen

for tissue engineering and Regenerative Medicine applications.[14, 15] These techniques allow the use of strong and physiological collagenous fibers/fibrils, but are limited by the control over the construct morphology. In contrast, a high degree of control has been demonstrated for a wide range of approaches by aligning soluble collagen molecules, including electrospinning[16, 17], magnetic patterning[18], printing[19], dip-pen (nano)lithography[20], shear flow patterning[21], microfluidic shear flow channels[22], salt and/or particulate leaching[23], electrical gradient[24] and others[25]. These techniques exploit the orientational control of collagen monomers during self-assembly to form aligned collagen fibrillar structures.[26, 27] Constructs produced from monomeric collagen allow for high precision and standardization, and further allow for the seeding of cells before the polymerization of the gel into its final form.[27] Pre-seeded collagen gels are amenable for engineering of several tissues with defined collagen architecture like blood vessels and heart valves.[26-28] However, despite promising results, constructs based on monomeric collagen suffer from non-physiological small fibril dimensions and poor mechanical strength.[3]

1.3 EXTRUSION TECHNOLOGY

Largely unknown to the tissue engineering and Regenerative Medicine community is the utilization of extrusion techniques to produce materials for soft tissue replacement. Extrusion is defined as "the act or process of shaping a material by forcing through a die".[29] This has been applied in the biomedical material industry to produce all sorts of synthetic polymer threads or tubing, e.g. sutures, slings and silicon tubing. Its application to collagen is limited to making collagen fibrils, by extruding either monomeric collagen solution or fibrillar collagen through a circular die into a fibril-forming solution to produce a collagen monofilament which can be further processed by braiding, knitted and/or bundling into a strand, fabric or large bundle.[30-32] Two groups reported the use of a commercially available extruded fibrillar insoluble collagen film with undefined fiber orientation for *in vitro* cell differentiation, treatment of burn wounds and myocardial infarction with varying results.[33-37] Collagen extrusion technology using either one rotating cone or independently counter-rotating cones to influence collagen alignment originates from the packaging and meat industry, where it was designed to generate edible food casings from e.g. bovine or porcine skin-derived collagen fibers (see Figure 1A and B).[38] By using different types of extruders, defined fiber alignment, including linear or interwoven "crisscross" collagen fiber orientation, can be achieved.[39] In this study, we applied state-of-the-art controlled fiber alignment extrusion technology, using counter-rotating cones, to develop a tubular collagen construct with defined morphology and fiber architecture.

2. MATERIALS AND METHODS

2.1 TISSUE PREPARATION

Porcine tissues (Achilles tendon, left subclavian artery and skin) were purchased from a local slaughterhouse and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 24 h at 4 °C. The samples were sliced with a vibratome (500 µm).[40]

2.2 COLLAGEN SOURCE

A 4% (w/v) collagen fiber suspension, or gel (Unicoll™813, Barentz Raw Materials, Hoofddorp, Netherlands), swollen in 1.0% (w/v) acetic acid and 1.5% (w/v) lactic acid (final pH 3), purified from bovine hide splits was used.[39, 41] In general, bovine hides are alkaline-dehaired (usually using 6–10% w/v calcium hydroxide) and the corium layer is split from the hide parts used for leather production (performed by tanneries). Subsequently, the corium layers are decalcified, ground into smaller particles, swollen in acid and finally homogenized under high pressure to produce an insoluble fibrous collagen dispersion. This dispersion is easily precipitated by the removal of water by using for example high concentration salts.[39]

2.3 FILM EXTRUSION

The collagen gel was mixed using a vacuum mixer (VM60A, Stephan, Hameln, Germany) for 30 min at 4 °C and subsequently loaded into the extrusion system.[42] The coextrusion system (Figure 1C) consists of a piston stuffer (FA30 TOP, Handtmann,

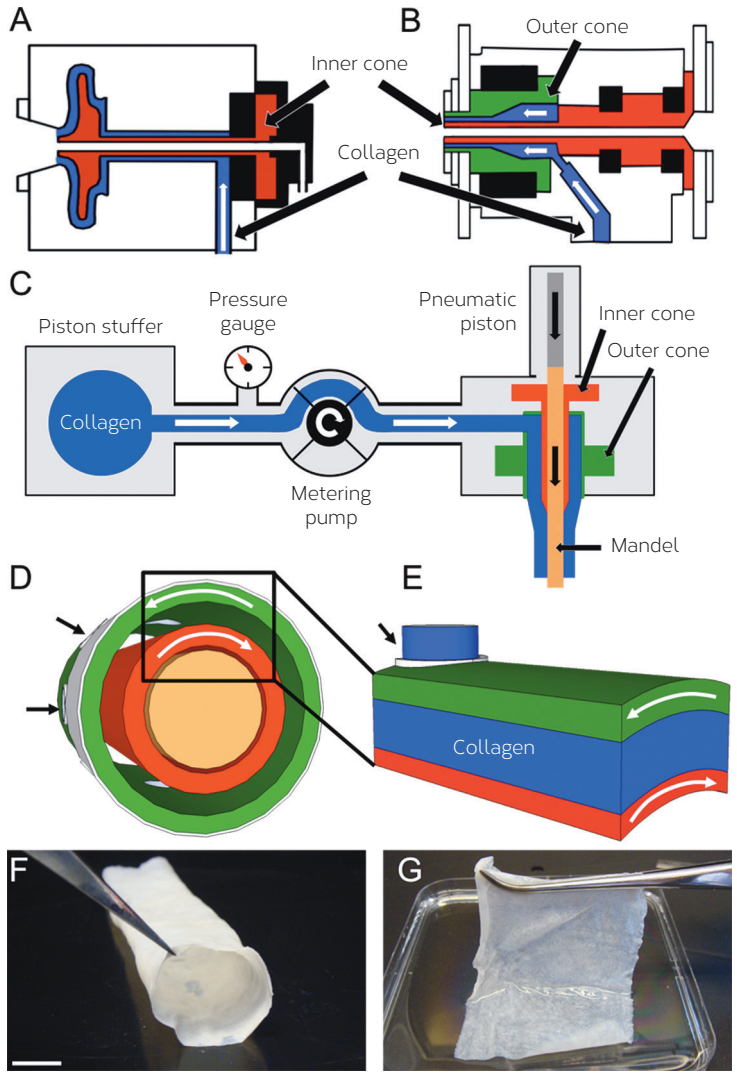

FIGURE 1: OVERVIEW OF EXTRUSION TECHNOLOGY AND THE SYSTEM SETUP USED.

A) Single cone extruder design and **B)** Counter-rotating cone extruder design used in this study for collagen extrusion. Either one or two rotating cones can be used to influence the fiber direction. The inner cone (red) and the outer cone (green) are the rotating parts between which the collagen gel (blue color) is extruded. White arrows show direction of collagen flow. **C)** Scheme of the collagen delivery setup, using 5 bar of pressure generated by the piston stuffer and defined flow rates controlled by the metering pump. Subsequently the collagen flows through the counter-rotating extrusion head onto a pneumatically controlled mandrel. White arrows represent collagen flow and black arrows represent extrusion direction of mandrel. **D)** and **E)** Cross-section of the extrusion head, with counter-rotating inner (red) and outer (green) cones molding the collagen film during extrusion. **F)** Tubular collagen construct, bar represents 1 cm. **G)** Tubular collagen construct cut open for further characterization.

Biberach, Germany), followed by a metering pump, extrusion head and pneumatic piston (all performed in a refrigerated conditions at 4 °C, Marel Townsend Further Processing, Boxmeer, Netherlands). Briefly, the piston stuffer applies a constant collagen pressure (5 bar) to the metering pump (55 rpm), which subsequently supplies the extrusion head (continuously cooled to 4 °C) with a 700 g collagen suspension per min. The interchangeable extrusion head (Figure 1D) was a 24 mm (inner Ø) extrusion head with independently counter-rotating cones. The collagen traveled from the pump through the rotating extrusion head, in between the inner and outer cone (Figure 1E), and was finally extruded through a 350 µm circular die. The collagen was deposited on a Ø 23 mm mandrel, which was forced out by a pneumatic piston at 80 mm s–1 in order to keep a constant traveling speed. After extrusion, the collagen-coated mandrels were placed in 6 M NaCl to set the collagen into a film. The films were stored in 6 M NaCl until further testing commenced. At least 20 mandrels were extruded, from which two films for texture analysis and one sample for microscopy were randomly taken per mandrel. The collagen films were maintained orthoptically with defined longitudinal and transversal directions up until second harmonic generation imaging (as described later in section 2.5).

2.4 MECHANICAL CHARACTERIZATION

The collagen films were removed from the mandrels and cut into 150 mm (length) × 60 mm (width) rectangles, whilst retaining the original extrusion orientation in the lengthwise (l) direction. To facilitate reproducible tensile strength testing, a 5 mm notch on each side in the middle of the length of the film was made using a scalpel and the film was subsequently stretched to failure to assess its mechanical characteristics (TA.XT Plus texture analyzer, Stable Micro Systems, Surrey, UK). The films were clamped between tensile grips and subjected to tensile analysis (test speed: 40 mm s–1; trigger type: button; 100 mm intra-clamp spacing; n = 15). The resulting tensile profiles were analyzed for maximum force (N), the slope (the average between 20 and 80% of the maximum force, corrected for film thickness to yield kPa) and the area under the curve (total work to rupture until maximum force, in mJ) using eXponent software (Stable Micro Systems). Films that tore at the site of the tensile grips were excluded from analysis. The thickness was assessed using a texture analyzer equipped

with a Ø 5 mm stainless steel cylindrical probe (test mode; compression, test speed; 0.05 mm s–1, post-test speed; 10 mm s–1, target mode; force, 1 N, trigger type; button, n = 40). Films were placed on a plate and approached with the probe. Upon reaching a force of 1 N, the remaining distance to the bottom plate was taken as the film thickness. All mechanical characterizations were performed on films removed from 6 M NaCl prior to each individual test.

2.5 SECOND HARMONIC GENERATION MICROSCOPY

Second harmonic generation (SHG) imaging was performed on an upright multiphoton microscope (TriMScope-II, LaVision BioTec) equipped with a 20× objective (Olympus XLUMPlanFl 20×/0.95 W, IR-enhanced), three tunable Ti:Sa laser systems (Chameleon Ultra I and II Titanium:Sapphire, Coherent Inc.) and an optical parametric oscillator (OPO PP Automatic, equipped with a PP800 crystal; coherent APE; typical pulse width 200 fs, repetition rate 80 MHz; output power 650 mW at 1090 nm).[43] Collagen films were excited at 850 nm with a constant power of 120 mW under the objective. The emission of the moderately transparent collagen films was collected in the forward direction using a 1.4 NA condenser, guided through a narrow bandpass filter (Semrock Inc., FF01 427/10) and detected with a blue sensitive photomultiplier tube (Hamamatsu, H6780-01). On the other hand, the more scattering and absorbing tissue slices and an accompanying reference collagen films were excited at 1090 nm wavelength with a linearly imaging depth-dependent power of 50–200 mW under the objective. The emissions of the 1090 nm excited samples were collected in the forward direction, filtered (Chroma Technology Inc., ET525/50) and detected using an ultrasensitive gallium arsenide phosphide photomultiplier tube (Hamamatsu, H7422-40). Collagen films were imaged in PBS solution (pH 7.4).

2.6 IMAGE ANALYSIS

Four mosaicked z-stacks were stitched together with a 10% overlap and 10 µm spacing between the slices using Fiji (version 1.47b) with the stitching plug-in.[44] Collagen fiber orientation was quantified per slice using Fourier transformation (Fiji directionality plug-in; Nbins: 90; histogram start: −90°).[45] The dominant fiber directions of the fitted Gaussian curves of the 200–200 rpm and 0–0 rpm speeds were plotted, after which the results were combined into one graph. Additionally, a trendline was made from each 200–200 repetition and combined into one main trendline.

2.7 MATHEMATICAL MODELING

Response surface methodology (RSM) was employed to investigate the effect of cone speed on fiber alignment.[46] RSM allows empirical modeling using the central composite design based on the two-variable inscribed model.[47] This second-order model allows simultaneous modification of two explanatory variables (inner and outer cone speeds). As upper and lower limits of each variable, mechanical limitations of the extrusion machine (Marel Townsend Further Processing, Boxmeer, Netherlands) for the relative inner (100–315 rpm) and outer cone (100–400 rpm) speeds were taken into account.[42] Inner and outer cone speeds of 200 rpm served as a central point (Table 1) using the "Design of Experiments" feature included in the XLSTAT software (Addinsoft, New York, United States).[47] Collagen extruded with both cones stationary (0–0 rpm) was used as the control. To visualize the effect of cone rotation, the collagen fiber direction was plotted against cone speed and a surface was fitted through the data using XLSTAT software (version 2013.4).

TABLE 1. SUMMARY MECHANICAL PROPERTIES OF EXTRUDED FILMS AT DIFFERENT SPEEDS

Outer Cone *rpm*	Inner Cone *rpm*	Thickness *mm*	Force at Break *N*	Work *mJ*	Young's Modulus *kPa*
300	300	0.20 ± 0.04	2.48 ± 0.46	34.3 ± 8.8	935 ± 210
	200	0.22 ± 0.03	2.27 ± 0.42	26.7 ± 6.7	866 ± 193
	100	0.20 ± 0.05	2.43 ± 0.46	23.3 ± 5.3	1257 ± 293
200	300	0.16 ± 0.04	2.10 ± 0.38	23.1 ± 6.3	1242 ± 162
	200	0.18 ± *0.01*	2.53 ± *0.11*	26.8 ± *0.7*	1283 ± *53*
	100	0.19 ± 0.05	2.42 ± 0.43	21.5 ± 5.2	1467 ± 314
100	300	0.16 ± 0.03	2.29 ± 0.32	22.3 ± 4.2	1497 ± 155
	200	0.17 ± 0.05	2.46 ± 0.41	19.3 ± 4.6	1925 ± 310
	100	0.17 ± 0.03	2.53 ± 0.50	19.2 ± 5.3	1948 ± 339

All values show average ± standard deviation unless stated otherwise
**Central point (200–200, n=5) in experiment design shows average ± st*andard error of mean

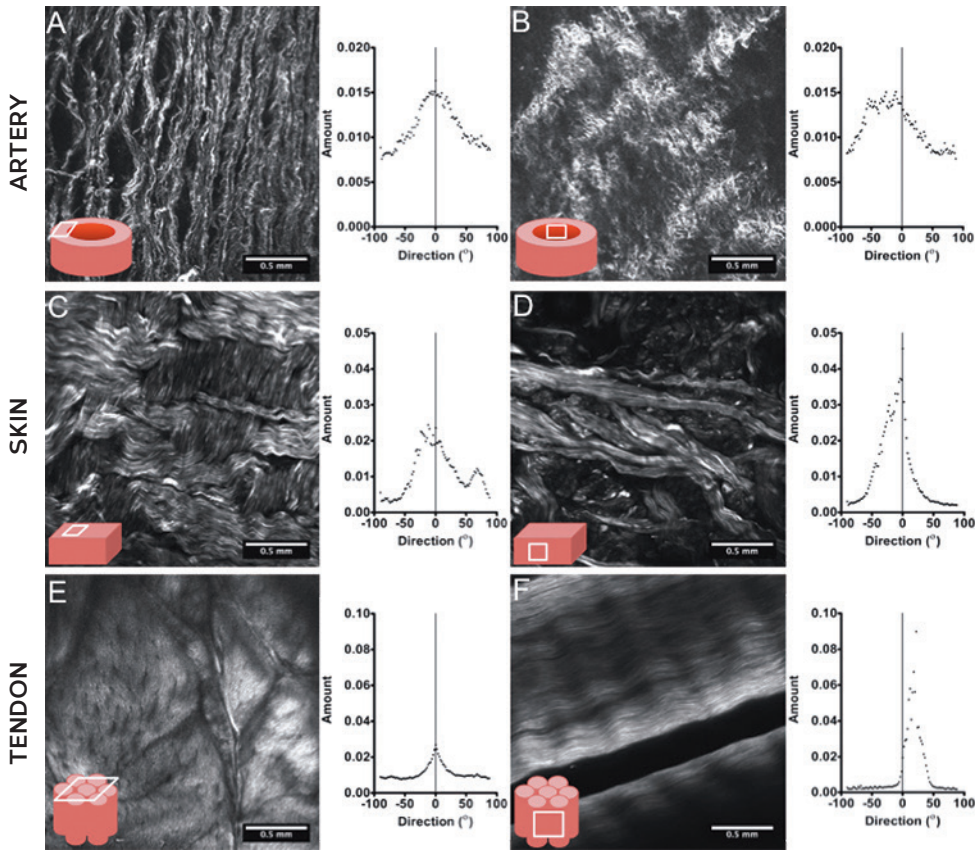

FIGURE 2: DIRECTION OF COLLAGEN FIBERS IN PORCINE COLLAGEN-RICH ORGANS.

Images from vibratome-sliced porcine tissue sections, using two-photon excited SHG microscopy to visualize collagen (white signal). Images were subjected to directional analysis of which results are displayed in graphs on the right. **A)** Tunica media layer of subclavian artery. **B)** Sub-endothelial matrix of subclavian artery. **C)** longitudinal and **D)** cross sectional view of dermal matrix of skin. **E)** cross section and **F)** longitudinal section of achilles tendon. Bars represent 0.5 cm.

3. RESULTS

3.1 FILM EXTRUSION

To produce tubular films, collagen gel was extruded via the counter-rotating cones onto the mandrels and subsequently set by NaCl precipitation. This resulted in a tubular film that could be removed from the mandrel in both the wet state (Figure 1F) and the dry state after freeze-drying. The construct was cut open to further facilitate mechanical and structural characterization (Figure 1G). The extrusion of films produced with stationary cones (0–0 rpm) did not yield a mechanically stable film on its own and was not viable for mechanical characterization.

3.2 DIRECTION OF COLLAGEN FIBERS IN TISSUES

To demonstrate the large differences in collagen architecture, its orientation was analyzed using SHG microscopy. Figure 2 shows an overview of the collagen fiber architecture in several organs. Circumferential collagen fibers in largely aligned orientation were present in the tunica media of the subclavian arterial wall (Figure 2A), whereas a more meshwork-like orientation with less defined directions was seen sub-endothelial (Figure 2B). In the dermis of the skin, the top view (Figure 2C) showed a woven collagen structure, whereas the cross-section showed the collagen fibers traversing different depths (Figure 2D). Two peaks that are approximately 90° apart can be seen in the directionality graph from the top view of the dermis, which indicates perpendicularly oriented collagen fibers. The Achilles tendon showed collagen fibers grouped in bundles (Figure 2E), the cross-section indicating the high degree of organization with the collagen fibers aligned in one direction (Figure 2F), with a very narrow distribution of the directionality peak.

3.3 COLLAGEN FIBER DIRECTION

The influence of the cone speeds on collagen orientation was investigated using SHG and image analysis. In Figure 3 an overview of images from speed 200 rpm (inner)–200 rpm (outer) are shown as an example. A selection of the slices is depicted

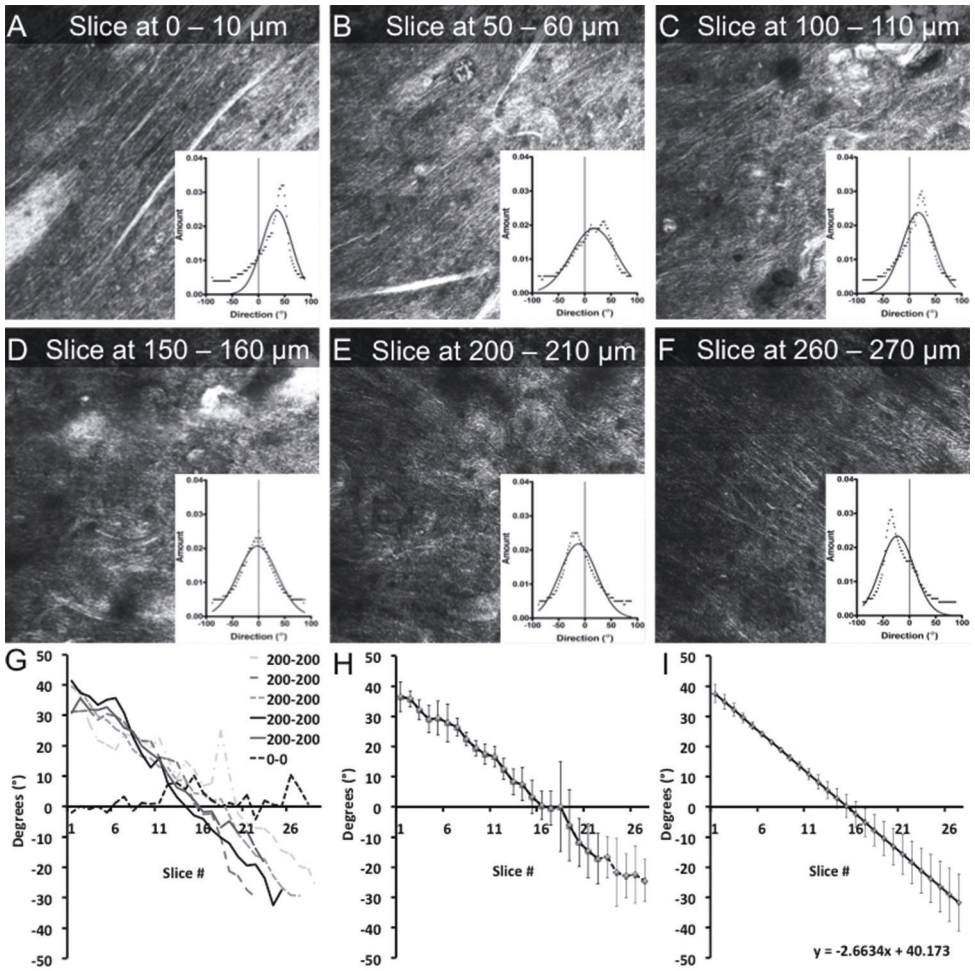

FIGURE 3: DIRECTIONAL ORGANIZATION OF EXTRUSION-GENERATED 3D COLLAGEN FILMS.

A–F) Second harmonic signal images and quantification of fiber orientation (graphs, inset) from individual slices obtained at indicated depth position of image stack from films generated at 200 - 200 rpm cone speeds. Along the x-axis, the fiber orientation, in degrees (°) is shown where the collagen fiber orientation in the slices is measured and subsequently fitted with a Gaussian curve (solid lines). The peak of the curve represents the dominant fiber direction. **G)** Dominant fiber directions from speed 200-200 plotted against a direction for 0 - 0 control cone speed. **H)** Average dominant fiber direction profile of all 200-200 speeds combined, error bars represent standard deviation. The graph's solid black line changes to a dotted line from slice 21-27, which represents a deviating number of available data points (lower than n=5). **I)** The trendline average from combined 200-200 cone speeds.

from which the dominant fiber direction is plotted. From these results we observed a general trend where the top part (close to the outer cone) of the sample reveals fiber directions ranging from 30 to 45°. This angle gradually changed to 0° at a depth of 150–200 µm (15–20 slices). Finally, the angle of the bottom part (close to the inner cone) of the sample changed from −20 to −35°. An overview of the fiber direction of the central point (200–200) is plotted in Figure 3G. When all the points were combined into one line (Figure 3H), the general trend become clear, the fiber direction decreasing by about 2.6° every 10 µm (Figure 3I). A control film made with stationary inner (0 rpm) and outer (0 rpm) cones showed a fiber direction corresponding to the extrusion direction (0°) throughout the whole film (dotted black line in Figure 3G).

3.4 MECHANICAL CHARACTERIZATION

To determine if the different cone speeds influenced the mechanical properties of the films, tensile strength experiments and thickness measurements were performed. The collagen films were found to have an ultimate tensile strength between 2.1 and 2.5 N, a work until fracture between 19.2 and 34.3 mJ, a Young's modulus between 886 and 1948 kPa and a thickness between 160 and 220 µm (Table 1). From the response surfaces, the total force necessary to rupture the films increased with higher cone speeds (Figure 4A). A clear pattern can be seen, with a combination of high inner and high outer cone speeds resulting in higher tensile work. Surface responses of the Young's modulus show that films made at both lower inner and outer cone speeds were more elastic (Figure 4B). In general, the collagen films made by using different cone speeds did not differ in their maximum tensile force, with a maximum tensile strength spanning 2.1–2.5 N. A slight trend could be found where films produced with a >250 rpm inner cone speed resulted in weaker films (Figure 4C). Furthermore, higher outer cone speeds resulted in thicker films. The inner cone speed did not contribute to the film thickness to the same magnitude as the outer cone (Figure 4D). The tensile testing results (force at break and work) were not corrected for the thickness as the thickness was inherent to the film produced at given cone speeds and is therefore part of its standard of identity.

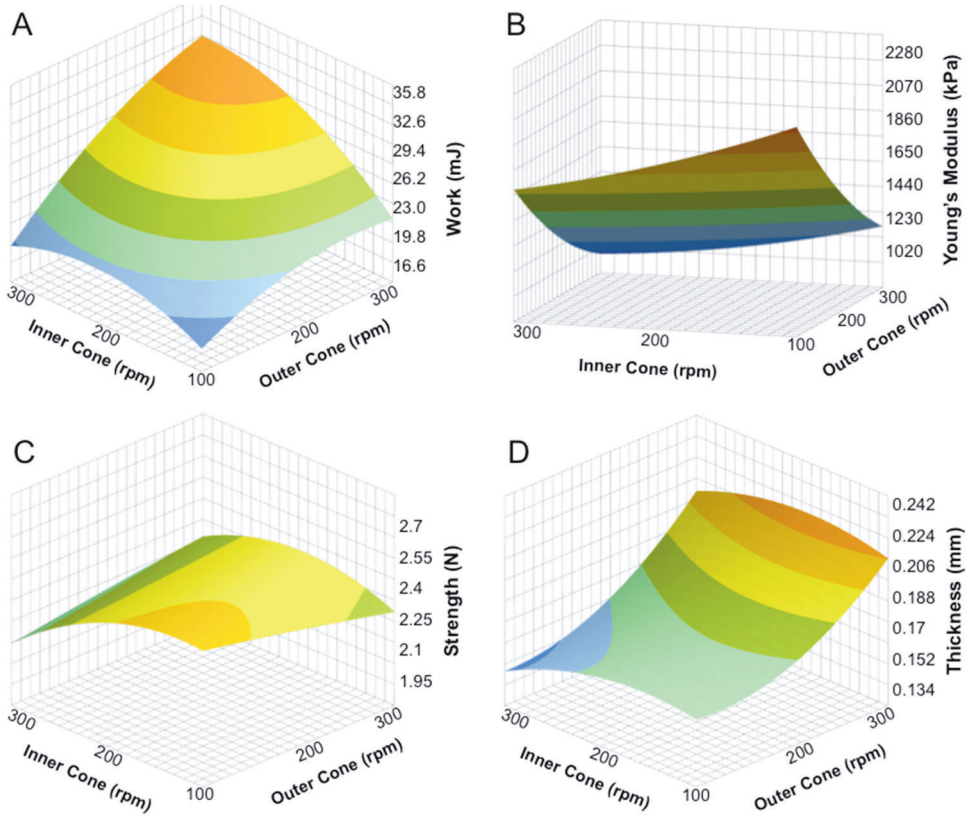

FIGURE 4: EFFECT OF EXTRUSION CONDITIONS ON MECHANICAL PROPERTIES OF THE EXTRUDED FILMS.

Surface response curves of the inner (x-axis) and outer cone (z-axis) speeds versus **A)** film toughness as determined by work. **B)** Film elastic modulus as determined by Young's Modulus on the y-axes. **C)** Film tensile strength. **D)** Film thickness.

4. DISCUSSION

4.1 COLLAGEN FIBER ALIGNMENT

We show here that the fiber direction of collagen tubes prepared from native collagen fibers can be influenced using shear force applied by independently counter-rotating cones. We utilized a nonlinear optical technique, SHG microscopy, to visualize the collagen fibers.[48] In the recent years, SHG microscopy has gained popularity in imaging thicker tissues, as the wavelength in the infrared spectrum can penetrate samples up to 1 mm.[49] Evaluation of the films using SHG microscopy revealed a general trend where the fibers in the top region of the films were oriented in the rotation direction of the outer cone. This was also true for the fibers in the bottom region, where the collagen was oriented in the rotation direction of the inner cone. The change in fiber direction seemed to be a gradual change throughout the sections of the sample, with an average decrease in 2.6° every 10 µm. Depending on the cone speed, the fibers tended to align more towards the nearest cone. In the case of static cones, the shear forces produced by the flow of the collagen result in films with fibers aligned in the flow direction. It is possible that the rotation of the inner and outer cone can only influence the collagen fiber alignment up to a certain extent (depth). In Figure 5 we present an overview of a tubular construct extruded using a counter-rotating cone system showing a typical fiber direction. Collagen fiber orientation on the outer layer follows the direction of the outer cone, whereas collagen fiber orientation on the inner layer follows the direction of the inner cone. The exact mechanism needs further elucidation, but it is possible that the influences of the forces produced by the outer and inner cone rotation gradually diminish towards the middle of the film, where the shear force of the flow direction is dominant. This notion is supported by the observation that all films show a gradual change in fiber angle. In a hypothetical situation where the shear force, caused by the flow is removed, the collagen fibers will eventually fully align in the rotation direction of the dominant cone. This phenomenon has been described previously for tubular scaffolds made by the insertion of a rotating mandrel in soluble collagen during the polymerization process.[21]

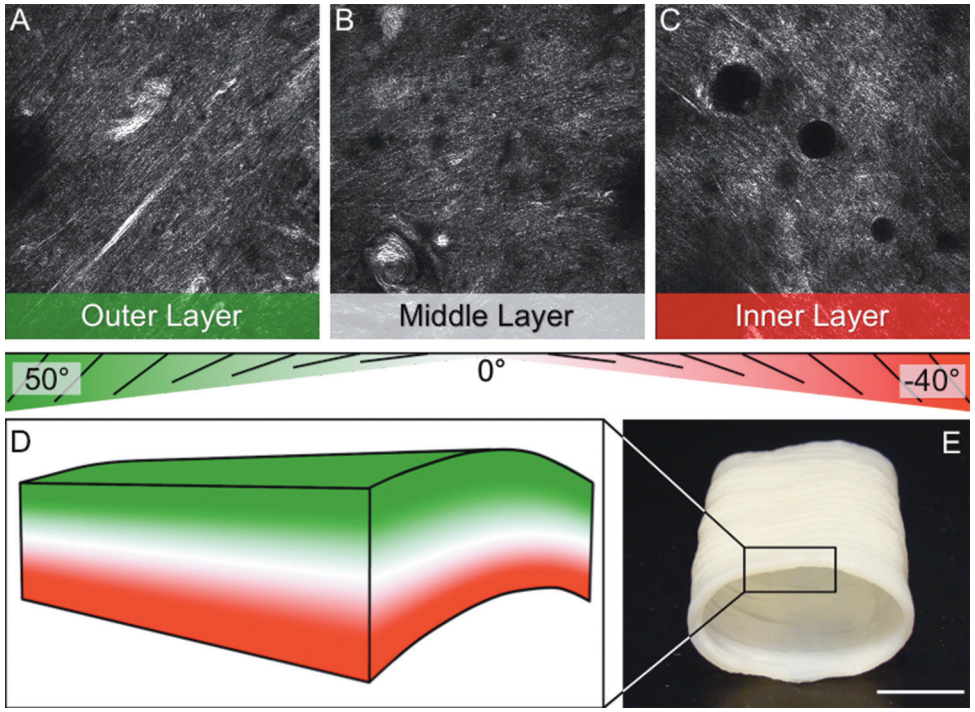

FIGURE 5: OVERVIEW OF DIRECTED ALIGNMENT OF COLLAGEN FIBERS USING EXTRUSION TECHNOLOGY APPLYING COUNTER-ROTATING CONES.

A–C) average two-photon images of the inner, middle and outer layer of an extruded film. **D)** Carbonized visualization of the general collagen fiber direction found in an extruded film derived from **E)** tubular construct. Bar represents 1 cm.

4.2 MECHANICAL PROPERTIES

The films were subsequently subjected to mechanical characterization (maximum tensile strength, work until rupture and elastic modulus). A clear trend can be seen where higher inner and outer cone speeds result in tougher films. The opposite is true for the Young's modulus of the films: lower inner and outer cone speeds result in films with a higher elastic modulus. Surprisingly, the trend of fiber alignment was similar in most tested conditions; however, it is unlikely that the difference in the mechanical properties can be correlated to these slight variations in fiber orientation. Conversely, the difference in the mechanical properties might be explained by the manner in which the collagen structures are intertwined or connected. Hypothetically, the collagen fibers exposed to more shear force could be less randomly coiled in higher structural hierarchies. Subsequently, the energy from the shear force exerted on the collagen could be stored in the form of tension between the collagen structures, ergo resulting in a tougher construct. The higher cone speeds may also result in a more homogeneous distribution of the collagen fibers that might account for the increase in toughness. The thickness of the films also showed variations to some extent. This may be caused by the method used to load the collagen into the extrusion head. Most probably due to centripetal forces, higher outer cone speeds seem to increase the pressure inside the extrusion head and subsequently increase the pressure exerted on the collagen. Despite the speed of the collagen dosage being kept constant by the metering pump, this effect may temporarily increase the collagen dosage during the extrusion process at higher cone speeds, resulting in thicker films.

4.3 EXTRUSION IN TISSUE ENGINEERING AND REGENERATIVE MEDICINE

Controlling the fiber direction and alignment, mimicking the ECM, is of great importance when designing biomaterials for tissue engineering applications.[50] The great difference in collagen architecture of each organ is demonstrated in Figure 2. Tissues carrying high loads have highly aligned collagen fibers, like in the tendon. The woven-like collagen morphology in the skin allows for bending, stretching and compression. Extrusion using a counter-rotating cone system allows us to influence

the fiber alignment of collagen-based tubular constructs, subsequently adding a novel method to the currently available tools to mimic the ECM of tissues. Moreover, by modifying the procedures of the extrusion approach, such as varying the speeds, pressure, density of the source of collagen and/or thickness of the extrusion channel, it may be possible to control the fiber characteristics, direction and variation thereof within the same film.[51] A point of attention in the future will be to adjust the current stiffness (900–2000 kPa) to the target tissue in question, which may be done by varying, for example, the collagen concentration, salt concentration, degree of crosslinking and incorporation of other biomaterials.[52] In addition, iterative passaging of films through the machine or several cones in series may be suited to generate distinct yet intertwined layers. Extrusion without rotating forces causes the fibers to align in the extrusion direction. Adjusting the composition of the materials that are extruded can also have a synergistic effect on cellular response. For example, the concentration and type of collagen can be changed to make the film more porous and subsequently allow for better cell penetration. Porogens and or soluble particulates can be added to increase porosity, if desired.[53] Elastin fibers and derivatives thereof can be added to improve smooth muscle cell growth and decrease thrombogenicity.[54,55] In order to further improve the mechanical properties, synthetic polymers in the form of knittings can be incorporated.[56] The extrusion system allows for complex scaffolds to be designed where each different application needs a tailored combination of film morphology, size and biological composition. For example, arteries or urogenital structures could be made using a 3–7 mm diameter extrusion head with specifically formulated inner and outer layers. Larger extrusion heads could be used to make scaffolds for applications in gastrointestinal repair or aortic replacements.[57] We believe that the novel collagen extrusion method presented here is a valuable addition to the scaffolding armamentarium to create a wide range of standardized tubular tissue equivalents under controlled and standardized circumstances.

5. CONCLUSION

Here we demonstrated that an extrusion system equipped with counter-rotating cones can produce collagen films in which the fiber orientation and subsequently the mechanical characteristics can be influenced. We consider this as enabling technology that represents a highly adjustable novel method able to produce single- and multilayered scaffolds. The method is suitable for large-scale and low-cost production of tubular tissue equivalents.

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CHAPTER 06

FUTURE BLADDER RECONSTRUCTIONS:

A SPHERICAL HOLLOW COLLAGEN BLADDER CONSTRUCT WITH APPENDICES

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IN CONSIDERATION FOR PUBLICATION

ABSTRACT

The field of Regenerative Medicine has developed promising techniques, which can help to improve current neobladder methodologies used for radical cystectomies or congenital anomalies. Scaffolds made from molecularly defined biomaterials are instrumental in the regeneration of tissues, but are generally confined to small flat patches and do not comprise the whole organ. We have developed a simple, one-step casting method to produce a seamless large hollow collagen-based scaffold, mimicking the shape of the whole bladder, and with integrated anastomotic sites for ureters and urethra. The hollow bladder scaffold is highly standardized, with uniform wall thickness and a unidirectional pore structure to facilitate cell infiltration *in vivo*. Primary bladder urothelial cells and smooth muscle cells were able to attach, proliferate and differentiate on/in the scaffold. The technology used is highly adjustable (shape, size, materials) and may be applied to produce off-the-shelf medical devices suitable for neobladders to treat cystectomy patients. A potential treatment modality using acellular scaffolds is discussed.

1. INTRODUCTION

In case of muscle-invasive and refractory superficial bladder cancer and end stage (congenital) bladder disease, the current clinical standard is radical cystectomy in combination with urinary diversion.[1, 2] The method of diversion depends on, amongst others, the nature of the defect, and the patient's needs and wishes. Orthotopic bladder reconstruction is increasingly applied for urinary tract reconstruction.[3] However, current methods rely on autologous tissues that are harvested from the gastrointestinal tract. This can lead to severe complications including anastomotic leakages, enteric fistulae, bowel obstruction, prolonged episodes of ileus, life-threatening infections, nutritional mal-absorption, and/or intestinal failure.[1, 4]

New techniques and materials generated in the field of Regenerative Medicine may provide useful alternatives. Regenerative Medicine (RM) aims to regenerate tissues and organs by creating biological equivalents through the supplementation of scaffolding materials, bioactive components, cells or a combination thereof.[5] Within the field of RM different attempts have been made to reconstruct the bladder in both animal and human studies.[6] In 2006 a promising avenue for RM in producing a neobladder was published by Atala *et al*. where a collagen/polyglycolic acid composite was used which was sutured together into a partial bladder/cup shape and seeded with urothelial and smooth muscle cells.[7] Initial clinical results were promising, but a recent related phase II clinical trial demonstrated that an autologous cell cultured scaffold composed of synthetic polymers did not improve bladder compliance and was associated with serious adverse events that surpassed the acceptable safety standard.[8] In addition, the complicated and expensive nature of the procedure may not be feasible in most clinical centers.[9-11] Alternatively, a well-structured molecularly defined acellular scaffold resembling the whole bladder may be an option, using the body as a bioreactor. Previously, flat acellular collagen scaffolds have been used for bladder augmentation in patients with exstrophy-epispadias complex and were found

to be completely lined with urothelial cells after implantation.[12] A tubular acellular collagen-based urostomy implanted in a pig model showed good results with respect to the re-urothelialization of the construct using the body as a bioreactor.[13] Flat scaffolds can be manually shaped into a sphere to create a bladder-like construct using sutures and a silicon breast prosthesis, as was shown by Baumert *et al*., who also pre-seeded the scaffold with urothelial and smooth muscle cells, and wrapped the construct in omentum for further cell differentiation *in vivo*.[14] Omentum wrapping of tubular acellular collagen scaffolds resulted in good vascularization and tissue integration of the scaffold.[15] Using bladder shaped acellular scaffolds for *in vivo* cellularization may prove to be effective for urinary diversions and neobladder reconstructions. However, recent proceedings from the "*2nd international consultation on bladder cancer: urinary diversion*", explicitly stated that widespread acceptance and success of a new technique is based on its simplicity.[16]

Despite promising results available for bladder engineering, it is clear that the approaches taken, including the culturing of autologous cells, are generally complicated, expensive and currently lack successful clinical trials.[9, 16] Therefore, new methods, including cell procurement and construct design, appear to be needed to advance this field. Taking this into account, we have focused on the design of a novel, simple, standardized and adjustable process to produce resorbable seamless hollow scaffolds that mimic the size and shape of a human bladder and include appendices for anastomosis of the ureters and urethra.

2. MATERIALS AND METHODS

2.1 CONSTRUCTION OF BLADDER SCAFFOLDS

A 0.7 % (w/v) suspension of highly purified type I collagen fibrils suspension in 0.25 M acetic acid (Scharlau, Spain) was prepared by overnight incubation at 4 °C.[17] The suspension was homogenized on ice using a Teflon glass Potter-Elvehjem device (Louwers Glass and Ceramic Technologies, Hapert, The Netherlands) with an intervening space of 0.35 mm (10 strokes). The suspension was deaerated by centrifugation at 117 g for 30 min at 4 °C. 500 mL of collagen suspension was poured into a custom-made aluminum (type 6082 T6) mold with a thermal conductivity of 205 W/m·K. The mold (Figure 1A) was specifically designed to mimic the shape of an adolescent human bladder, where three appendices were included to provide anastomosis sites for the ureters and urethra. The mold containing 500 mL collagen suspension was placed in a computer-controlled freezing bath (Proline RP890, Lauda GmbH, Lauda-Königshofen, Germany). To obtain a scaffold with a wall thickness of 10 mm, the mold was frozen at -20 °C for 30 min or -73 °C for 12 min, respectively, after which non-frozen collagen was removed from the mold (a schematic representation can be found in Figure 1B and 1C). The mold was subsequently placed in a -20 °C freezer for complete solidification of the frozen and non-frozen collagen interface. Frozen constructs were lyophilized in a freeze dryer (Sublimator 500 II, Zirbus, Bad Grund, Germany) and subsequently γ-irradiated (25 kGy, Synergy Health B.V., Ede, Netherlands).

2.2 TEMPERATURE MEASUREMENTS

Temperature differences in the collagen suspension were measured during the freezing process in triplicate using two thermocouples (Testo 922, Testo AG, Lenzkirch, Germany). Both sensors were placed in the bottom of the aluminum mold in the collagen suspension, where one sensor was placed on the aluminum surface and the other sensor 5 mm above it. The mold containing the collagen suspension was equilibrated at 4 °C, before transferring the mold to the computer-controlled freezing bath, which was set at -20 °C or -73 °C. Temperature measurements at -20 °C or -73 °C were performed for 30 min and 10 min, respectively, and the temperature was recorded with time intervals of 1 min. Cooling rates were calculated from the slope from 0 °C until the end of the measurement. The results are shown as mean ± standard deviation in °C/min.

2.3 SCAFFOLD CHARACTERIZATION

Magnetic resonance imaging (MRI; 11.7T animal scanner (BioSpec, Bruker, Germany)) was used to visualize the hollow structure of the scaffold with the following settings: Turbo-RARE pulse sequence; 37 ms echo time; 1500 ms repetition time; 180° flip

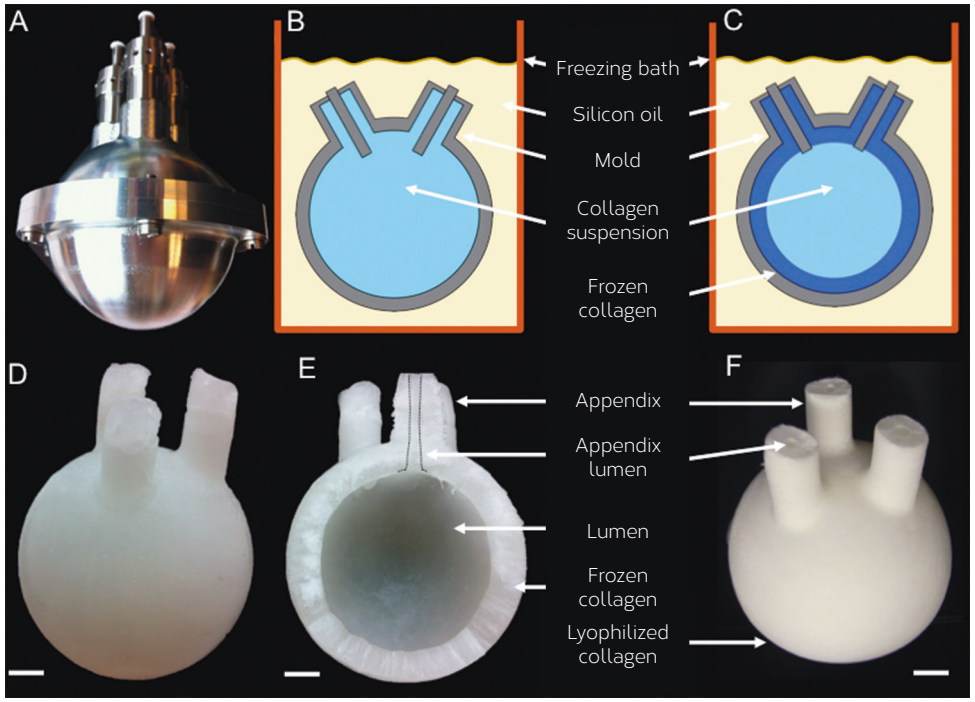

FIGURE 1: CONSTRUCTION AND MORPHOLOGICAL EVALUATION OF WHOLE BLADDER SCAFFOLD WITH APPENDICES.

A) Custom-made aluminum mold; **B)** and **C)** schematic representation of the freezing process and strategy to produce a seamless hollow construct with appendices. The mold was completely filled with a collagen suspension and placed in a computer-controlled freezing bath. After 30 min or 12 min freezing at respectively -20 °C and -73 °C, the non-frozen collagen was removed from the mold; **D)** frozen hollow collagen construct; **E)** frozen hollow collagen construct cut in half to display the hollow inside; **F)** freeze-dried scaffold which could easily be removed intact from the mold. Bars represent 1 cm.

angle; 0.195 x 0.195 x 1.5 mm/pixel spatial resolution; 12 min total acquisition time. Using ImageJ (1.47i, Wayne Rasband, National Institutes of Health, USA), the slices were subsequently combined to generate a 3D model.

2.4 PORE SIZE QUANTIFICATION

Scanning electron microscopy (SEM, JEOL SEM6340F, Tokyo, Japan) was used to characterize the intrinsic morphology of the scaffolds. Samples were mounted on stubs and sputtered with an ultrathin layer of gold using a Polaron E5100 Coating System. Images were recorded at an accelerating voltage of 10 kV. For both freezing temperatures, three bladder scaffolds were constructed by freezing and lyophilization, and evaluated. Punches were taken from the scaffolds from 14 different locations (bottom: 1; bottom-middle axis: 4; middle axis 4; middle-top axis: 4; top: 1), followed by crosslinking using vapor fixation with 37% formaldehyde under vacuum for 30 min.[18] From these punches, longitudinal and cross-sections were taken, and the inside and outside were evaluated. The longitudinal orientation was used to assess pore morphology. The cross-sections were used to investigate the pore size. Four images were recorded per cross-section and the lengths of the shortest axis of 40 pores per location were measured using the ImageJ trace tool.

2.5 CELL CULTURE

Mature porcine bladders were obtained from a local slaughterhouse and primary urothelial (pbUCs) and smooth muscle cells (pbSMCs) were isolated as previously described.[19, 20] After isolation, the cells were expanded for no more than 3 passages. Ø35 mm punches were excised from the large hollow spheres, washed with PBS and subsequently pre-incubated overnight with the respective media. pbUCs were cultured in keratinocyte serum-free medium (Life Technologies, Carlsbad, USA) supplemented with 50 µg/mL bovine pituitary extract, 5 ng/mL epidermal growth factor, 30 ng/mL cholera toxin (all Sigma Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Life Technologies). pbSMCs were cultured in smooth muscle cell medium (ScienCell, Carlsbad, USA) supplemented with 2% fetal bovine serum, smooth muscle cell growth supplement and 100 u/mL penicillin and 100 µg/mL streptomycin

(all Life Technologies). Before seeding, the medium-soaked scaffolds were placed on a ~1 cm stack of sterile Whatmann paper for about 5 min to remove excess medium, after which the scaffolds were transferred to a fresh stack. Then the cells were seeded on separate scaffolds (2.5 – 5 x 10^6 cells at a concentration of 5x10^6 cells/mL) where the pbSMCs were seeded on the outside of the scaffold and the pbUCs on the luminal sides. The cell suspension was allowed to sit on the scaffold for maximally 5 min after which the scaffold was carefully transferred to a 6-well plate with 2 mL medium. After all scaffolds were seeded, 4 mL medium was added until the scaffold was completely immersed. The seeded scaffolds were harvested after 1 and 7 days of culture. Samples were taken for immunohistochemistry and scanning electron microscopy. As a reference the pbSMCs and pbUCs were also seeded on glass slides and cultured for 7 days.

2.6 SCANNING ELECTRON MICROSCOPY AND IMMUNO-HISTOCHEMISTRY OF CELL-SEEDED SCAFFOLDS

For scanning electron microscopy, cultured samples were fixed using 2% (w/v) glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.4). Hereafter, the samples were washed with 0.1 M phosphate buffer for three times and subsequently dehydrated using ascending series of ethanol solutions (30, 50, 70 and 100%). Samples were dried using a critical point dryer (Polaron, Quorum Technologies, Rignmer, UK) using liquid CO_2 and imaged as previously described. For immunohistochemistry the samples were placed in Tissue-Tek (Sakura, Torrance, USA) and frozen in dry-ice cooled 2-methylbutane (Sigma-Aldrich, St. Louis, USA), sectioned (5 µm) using a cryostat microtome (Heidelberg, Heidelberg, Germany), mounted on superfrost slides (Thermo Scientific, Menzel GmbH & Co KG, Braunschweig, Germany) and stored at -80 °C until use. Subsequently, cytokeratin 7 (CK7) and α-smooth muscle actin (αSMA) immunofluorescent stainings were performed to identify the different cell types (see Table 1 for exact antibody types, dilutions and manufacturer information). In general, before staining, the slides were fixed using ice-cold acetone (-20 °C) for 10 min. The slides were blocked with 1% (w/v) normal goat serum in PBS for 10 min. Slides were incubated for 2 h with the primary antibody and for 30 min with the secondary antibody. After each incubation step, the slides were washed three times in PBS for 5 min. As a control, slides were incubated with the secondary antibody only. After staining, coverslips were mounted using wet mounting medium (Dako, Glostrup, Denmark). Cells cultured on glass slides were subjected to the same staining protocols.

TABLE 1. ANTIBODY SPECIFICATIONS

Antibody Specificity	Host	Clone	Provider	Dilution
Alpha Smooth Muscle Actin	Mouse	sp6	Thermo Scientific	1:8000
Cytokeratin 7	Mouse	OV TL 12/30	BioGenex	1:400
Collagen Type 1	Rabbit	AB746P	Merck Millipore	1:200
Mouse IgG heavy chain	Goat	N/A	Invitrogen	1:200
Rabbit IgG heavy chain	Goat	N/A	Invitrogen	1:200

2.7 STATISTICS

Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc., version 5, La Jolla, CA, USA). The effect of the freezing temperature on pore size was assessed by a two-tailed t-test. Pore sizes are shown as mean ± standard deviation. P-values < 0.05 were considered to be statistically significant.

3. RESULTS

3.1 SCAFFOLD CONSTRUCTION AND MACROSCOPIC EVALUATION

A collagen-based seamless hollow scaffold was constructed by freezing of a collagen suspension in a custom-made mold and lyophilization (Figure 1A). The freezing process yielded a mechanically stable construct, where the outside was frozen (Figure 1D). After removing the non-frozen fraction, the frozen part remained and had a hollow lumen (Figure 1E). In the frozen construct the lumen of the appendices appeared to be clear of any collagen residues. After lyophilization

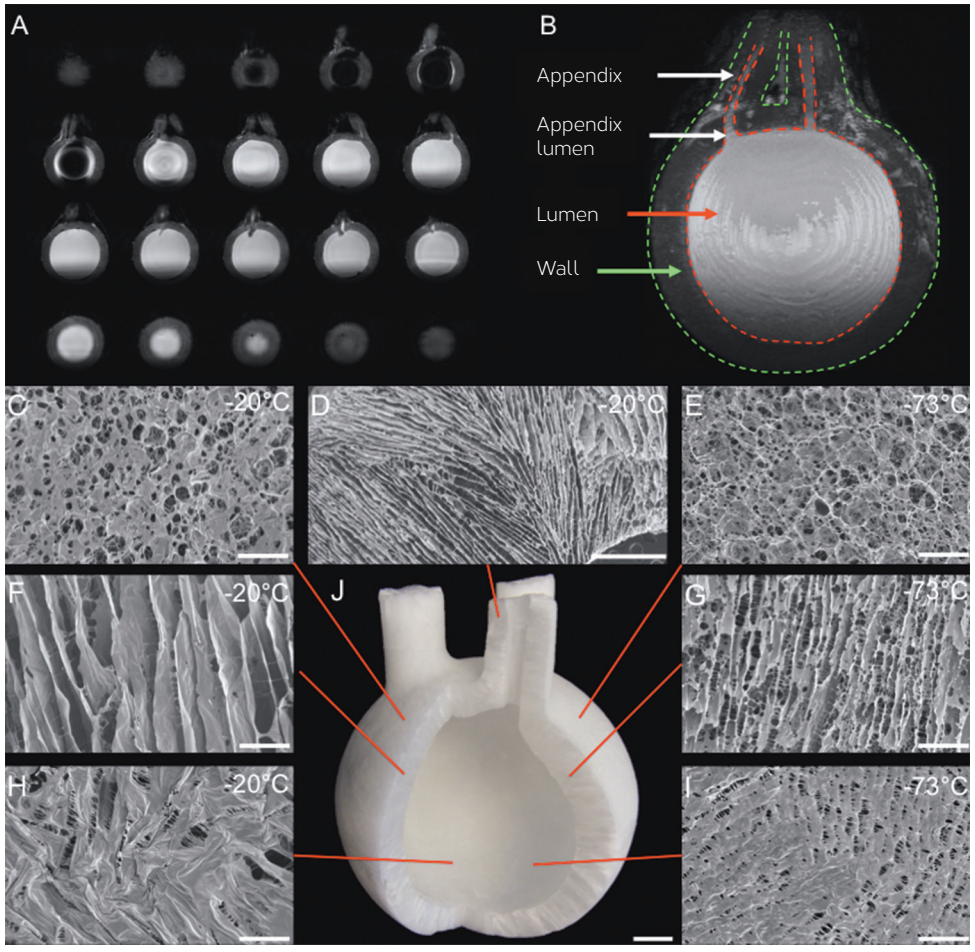

FIGURE 2: OVERVIEW OF THE BLADDER SCAFFOLD AND ITS STRUCTURAL CHARACTERISTICS.

A) MRI slices of the bladder scaffold; **B)** 3D model based on MRI slices. C-I) SEM images of the bladder scaffold; **C)** and **E)** exterior; **F)** and **G)** cross-section of the wall; **H)** and **I)** lumen; **D)** cross-section of the appendix. J) Macroscopical image of the bladder construct cut open. **A, B, D, F** and **H** were frozen at -20 °C and C, E and G were frozen at -73 °C. All bars in SEM micrographs represent 200 µm and macroscopical image bar represents 1 cm.

the collagen scaffold could be removed from the mold (Figure 1F). The inside of the spherical part of the scaffold was hollow and the surface had a homogenous structure. Moreover, the lumens of the appendices remained hollow and straight. The appendices were firmly and seamlessly attached to the spherical part of the scaffold.

3.2 SCAFFOLD STRUCTURE CHARACTERIZATION AND FREEZING MECHANISM

Magnetic resonance imaging (MRI) was used to visualize scaffold structure and generate a 3D model of the scaffold. The overall shape of the scaffold, wall, lumen, and appendices with their lumen are easily distinguishable (Figure 2A and 2B). The volume of the sphere was calculated using the MRI images and was determined to be approximately 380 ml. SEM images of different locations in the scaffold (spherical part and appendices) revealed a unique 3D unidirectional pore structure for scaffolds made at both -20 °C and -73 °C. The lumen showed low porosity with only few open structures whereas the outside of the scaffold was porous (Figure 2 C-I). The radial pore structure has been observed in other collagen scaffold types and is the result of the inward growth of ice crystals during the freezing process.[18, 21] Furthermore, the scaffold dimensions were charted where the wall thickness of the spherical part of the scaffold was 11 ± 2 mm and 8 ± 1 mm, whereas the thickness of the appendices was 6 ± 1 mm and 5 ± 1 mm for freezing at -20 °C and -73 °C, respectively. The inner diameter of the appendices was respectively 5 ± 1 mm and 6 ± 0 mm for freezing at -20 °C and -73 °C. The freezing process was characterized by temperature measurements. For both freezing temperatures, the sensor placed on the aluminum surface always showed a lower temperature compared to the sensor located 5 mm above the aluminum surface. This indicates a temperature gradient inwards; the freezing process started at the aluminum surface and progressed through the collagen suspension. Freezing at -20 °C and -73 °C resulted in cooling rates of 0.45 ± 0.01 and 3.84 ± 0.38 °C/min, respectively (Figure 3A). Freezing at different temperatures resulted in scaffolds with different pore sizes, the faster freezing protocol resulted in smaller pores (Figure 3B). Freezing at -20 °C and -73 °C yielded pore sizes of respectively 70 ± 17 µm and 43 ± 10 µm for the spherical part ($p < 0.0001$) and 74 ± 21 µm and 41 ± 12 µm for the appendices ($p < 0.0001$).

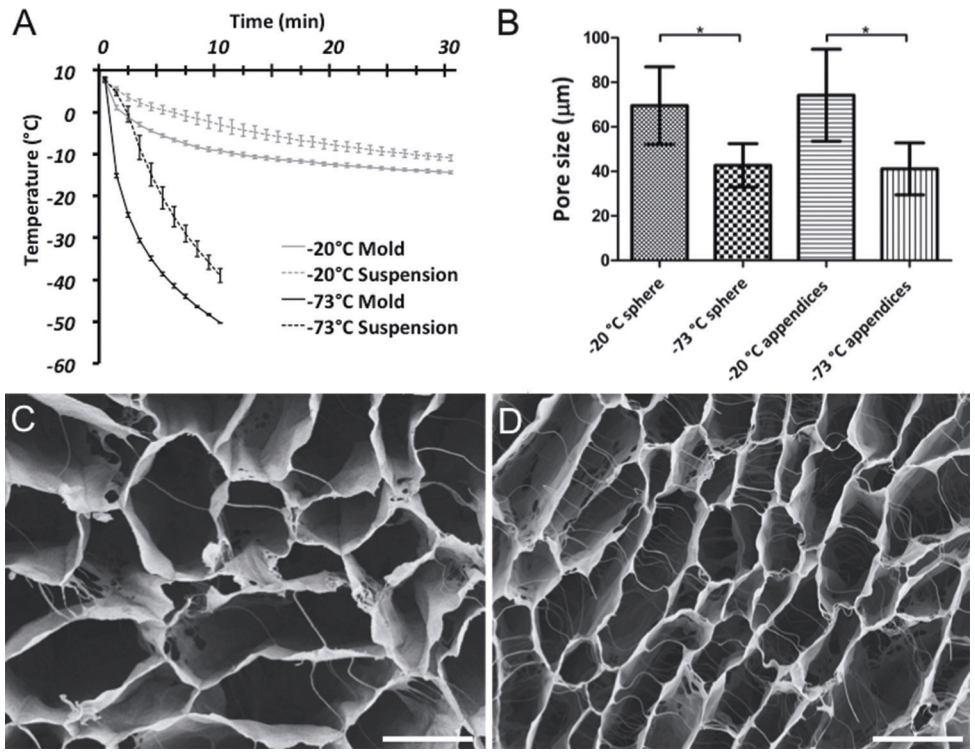

FIGURE 3: FREEZING CHARACTERISTICS AND PORE SIZE QUANTIFICATION.

A) Temperature of the collagen suspension during freezing at -20 °C and -73 °C. One sensor was placed on the inner surface of the aluminum mold and the other sensor was placed in the collagen suspension 5 mm above the aluminum surface. **B)** Pore sizes of the spherical part and the appendices of the collagen scaffold constructed using freezing temperatures of -20 °C and -73 °C, * $p > 0.0001$; **C)** and **D)** Pore morphology in cross-sections of scaffold prepared using freezing at -20 °C **(C)** and -73 °C **(D)**. The scale bars in SEM images represent 100 µm.

3.3 *IN VITRO* EVALUATION AND IMMUNOHISTOCHEMISTRY

To evaluate the cytocompatibility, pbSMCs and pbUCs were separately cultured on the luminal side and the outer side of the bladder construct, respectively, for 7 days, using excised Ø35 mm samples. In general, the cells were able to attach, proliferate and retain their phenotype on/in the scaffold. A confluent urothelial layer was observed as shown by SEM, and CK7 staining indicated the epithelial phenotype (Figure 4A-C). A confluent layer of striated pbSMCs was seen with SEM and these cells were positive for αSMA (Figure 4D-F). The pbSMCs formed multilayers but did not penetrate throughout the entire scaffold. Reference stainings were performed in order to evaluate antibody specificity (Figure 4G-L). Native porcine bladder epithelium was positive for CK7 before and after isolation (Figure 4G and 4H). The smooth muscle layer in the bladder was positive for αSMA (Figure 4J and 4K). The scaffolds seeded with pbUCs were positive for cytokeratin 7 (Figure 4B) but negative for αSMA (Figure 4I). Vice versa, scaffolds seeded with pbSMCs were positive for αSMA (Figure 4E) and negative for CK7 (Figure 4L).

4. DISCUSSION

RM-based methodologies to improve (re)construction of urinary reservoirs have a long and relatively unfruitful history. The complexity of the envisioned procedures for engineering complete bladders has hampered implementation in general.[11,16] Taking this into account we have designed a novel, simple, reproducible and adjustable process capable of producing a resorbable seamless hollow scaffolds that mimic the size and shape of a human bladder and include appendices for anastomosis of the ureters and urethra. The casting and freezing method is highly flexible with respect to scaffold size, volume, wall thickness and pore size, and can be adjusted by changing mold dimensions and freezing conditions. SEM analysis revealed a closed lumen for a urothelial lining and a porous exterior, which may, together with the unidirectional structure of the inside of the scaffold favor *in vivo* infiltration of cells and nutrients, and create a surface for a urothelial lining.

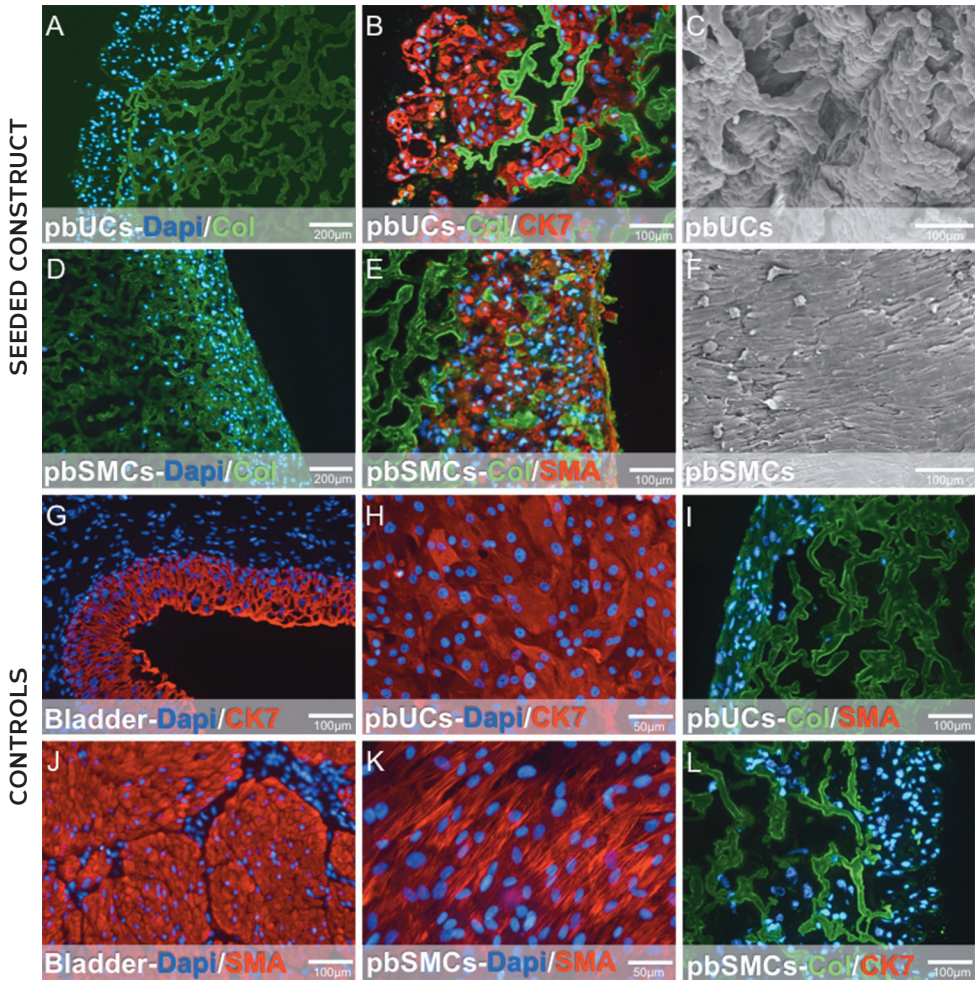

FIGURE 4: BLADDER SCAFFOLDS AFTER 7 DAYS OF CULTURE WITH UROTHELIAL OR SMOOTH MUSCLE CELLS AND OVERVIEW OF REFERENCE STAINING.

A) Overview staining of scaffolds seeded with porcine bladder urothelial cells (pbUCs), stained for type I collagen (green) and nuclei using DAPI (blue); **B)** pbUCs show positive staining for cytokeratin 7 (CK7, red); **C)** SEM micrograph of pbUCs showing a typical cobblestone morphology indicative for urothelial cells; **D)** overview staining of scaffolds seeded with porcine bladder smooth muscle cells (pbSMCs), stained for type I collagen (green) and nuclei using DAPI (blue); **E)** pbSMCs show positive staining for α-smooth muscle actin (αSMA, red); **F)** SEM micrograph of pbSMCs showing a stretched and aligned morphology. $5x10^6$ cells were seeded in **A, B** and **D**. $2.5x10^6$ cells were seeded in **C, E** and **F**. Staining of porcine bladder and subsequently isolated cells with CK7 (**G** and **H**) and αSMA (**J** and **K**). As a control, scaffolds seeded with either $5x10^6$ pbUCs or pbSMCs were both negative for **I**) αSMA; or **L**) CK7, respectively.

Recent advances in biomaterials for bladder tissue engineering have shown promising results, but still are often executed on a smaller scale with flat constructs.[22-24] The versatility of the hollow spherical construct described in this publication may prove to be a useful basis for other strategies currently being developed, where other materials may be incorporated into the casing technique to change the material properties (e.g., mechanical strength or degradation rate) if necessary.[15, 25] Advances within the procurement of the autologous cells from non-bladder sources (e.g., adipose and bone marrow tissue) for eventual seeding of bladder constructs may improve clinical outcome of future attempts, but are still laborious and costly.[26-28] Fortunately, techniques requiring minimal manipulation related to cell harvesting and seeding, that are simple but yet more effective, are gaining interest.[29] Despite increasing overall complexity of a clinically applicable procedure, bioreactor culture could be necessary in early stages of development and further *in vitro* optimization.[30, 31]

However, when keeping clinical application in mind, an acellular approach would improve overall clinical and commercial feasibility of first generation engineered bladders. To circumvent labor intensive and error-prone cell culture and seeding steps, the simplicity of the hollow spherical scaffold may be combined with existing straightforward techniques such as omental pre-implantation. For larger organs such as the bladder, omental pre-implantation appears to be the only route for effective and successful construct vascularization and subsequent prevention of fibrosis.[14] A recent study using tubular collagen constructs, omental wrapping proved to be instrumental in providing good vascularization and integration of the scaffold with omental tissue.[15] In case if cell seeding proves to be inevitable, seeded urothelial cells have been shown to survive and proliferate, even if omentum wrapping is applied.[20] Additionally, evidence suggests that the construct may be re-epithelialized from urothelial tissue in the ureters or remaining bladder tissue if an acellular approach is pursued.[12, 13] The increasing understanding of the bioactive signaling within the bladder ECM may also be combined with this approach and could increase the success-rate of an acellular approach.[32, 33]

We anticipate that the open directional pore structure of our scaffold will facilitate rapid ingrowth of vascular structures after omental preimplantation leading to additional surgical options next to the traditional approaches.[15] We envision a two-stage procedure consisting of pre-implantation/omental wrapping of an

off-the-shelf hollow scaffold several weeks before the planned cystectomy, to achieve vascularization. During cystectomy, the surgeon can utilize the pre-implanted scaffold directly, and opt to use it as a neobladder or to surgically adapt the construct to form an Indiana pouch-like urostomy.[2, 34, 35] This strategy will circumvent the complications from harvesting intestinal tissue and subsequent lengthy hospitalization.[1] Taking the simplicity of this technique into account, we foresee that, with extra development and input from the biomaterials community, the current scaffold could be a first step toward a standardized and cost effective approach for off-the-shelve next-generation neobladders. Whilst acknowledging that the full potential of the scaffold construct and associated surgical techniques needs extensive exploration, including investigating the *in vivo* efficacy of this strategy, a seamless hollow spherical scaffold may prove to be instrumental in the future of bladder engineering.

5. CONCLUSIONS

In this paper, a novel casting methodology was developed that resulted in a highly standardized collagen-based bladder scaffold with appendices, which is both easy to produce and highly customizable. This off-the-shelf construct may lead to a cheaper and flexible alternative to *in vitro* cell seeded engineered bladders.

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CHAPTER 07

DESIGN OF AN ELASTICIZED COLLAGEN SCAFFOLD:

A METHOD TO INDUCE ELASTICITY TO A RIGID PROTEIN

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IN PREPARATION

ABSTRACT

Mimicking the innate and complex mechanical properties of tissues is one of the major challenges the tissue engineering and Regenerative Medicine community faces in soft tissue repair. Mechanical discrepancies between the biomaterial in question and tissue to be repaired are a major contributor to graft failure *in vivo*. In this manuscript we describe a method where porous insoluble collagen type I scaffolds were compressed, corrugated and subsequently crosslinked using a carbodiimide-based crosslinking procedure. This resulted in a dense collagen scaffold with elastic-like properties and a pleated/corrugated wall structure. The scaffolds could be unfolded without an immediate increase in strain, after which the scaffold spontaneously returned to the relaxed corrugated position. Further mechanical characterization revealed that elastic-like characteristics were retained after 1000 cycles of unfolding and refolding (stretch-relax cycle). Cytocompatibility was tested by means of culturing fibroblasts on the collagen scaffolds. Histology and cell viability assays suggested that corrugated scaffolds possessed similar characteristics as their porous counterpart. By incubations of the corrugated scaffolds in solvents of various relative polarities, the mechanism behind the elastic-like characteristics was partially elucidated. The authors hypothesize that certain hydrophobic regions are introduced by the crosslinking of dense collagen in a fixed position. After exerting energy in the form of tension, the scaffold transitions into an energetically unfavorable state, which provides a counter force to return to its relaxed state. This methodology may in the future be applied to new and existing biomaterials to fine-tune mechanical and morphological properties to the target tissue. Overall, the methodology to elasticize a collagen construct could be a valuable addition to the scaffolding armamentarium for tissue engineering and Regenerative Medicine.

1. INTRODUCTION

Regenerative Medicine (RM) is based on combining knowledge gathered from the fields of molecular life sciences, cellular biology, biomedical engineering, (bio)materials science, reconstructive surgery and transplantation biology, to develop biomedical devices and treatments which aid in the repair or replacement of damaged tissues and organs.[1] More specifically, these biomedical devices or treatments can be the supplementation of vital cells, biomaterials, biomolecules or a combination thereof to the damaged site. This central dogma has lead to the development of biomaterials that mimic the extracellular matrix (ECM) and the strategy is based on stimulating the body's natural wound healing mechanism by implanting natural or synthetic ECM materials into a defect with or without cells. The ECM essentially acts as a template in the early stage of regeneration for cells to adhere or migrate to, proliferate and differentiate. Ideally, this hinders the formation of scar tissue and subsequently stimulates formation of new functional tissue, thus regenerating the defect.[2] These extracellular matrices, also referred to as scaffolds, can be classified into two groups: the decellularized tissues or molecularly defined constructs comprised of natural and/or synthetic biomaterials.[3, 4] For treatment modalities to succeed, biomaterial properties like bioactivity, biomechanics and overall morphology should be adjusted to that of the target tissue.[5, 6]

The mechanical properties of tissues or organs are largely determined by the composition of the ECM. The ECM is a dynamic and multifarious network that surrounds cells, providing structural and mechanical support in all tissues, mediating diverse biological processes that are crucial for supporting tissue formation and function, and playing an important role in wound healing. Major ECM components in tissues and organs include collagens, elastin, laminins, fibronectins, proteoglycans and glycosaminoglycans.[7] For example, organs that have load bearing functions like bone, cartilage, tendons and ligaments often have ECM's which are either rich

in collagen, minerals or a combination thereof.[8] Organs with functions that require repetitive motions like in the skin, lung, ligaments, blood vessels, trachea and the diaphragm often contain both elastin and collagen as the main structural ECM components.[9] Collagen provides tissues with essential tensile strength, enabling resistance to plastic deformation and rupture.[10] Type I collagen is one of the most frequently used naturally occurring biomaterial due to its ubiquitous presence in the ECM, superior biological properties and high mechanical strength for a protein. Collagen is naturally intended to add cohesiveness and rigidity to the ECM. Elastin on the other hand, provides tissues the properties of extensibility and reversible recoil, enabling tissues to withstand repetitive mechanical stress, and, this natural shape memory polymer is found in organs where shape and deformation recovery are crucial.[11] Moreover, in combination with these structural proteins, proteoglycans and glycosaminoglycans play an important role in the maintenance of optimal visco-elastic behavior, compressive stiffness and tissue hydration by sequestering water.[12]

Mimicking the innate and complex mechanical properties is one of the major problems the Regenerative Medicine community faces in soft tissue repair.[13] Mechanical discrepancies between the biomaterial in question and tissue for repair is a major contributor to graft failure *in vivo*.[14] The mechanical properties of biological tissues are considered to be highly dissimilar to most known biomaterials.[15] Elastic materials made from ECM components found in humans are underrepresented in current research efforts. Using ECM components offers several advantages over synthetic materials with respect to biocompatibility and bioactivity. ECM-based biomaterials generally have excellent biological properties but are mechanically dissimilar to the target tissue.[15] Collagen is frequently used to provide mechanical strength in scaffolds, however, depending on the scaffold type, both soluble and insoluble collagen-based scaffolds are often too weak (porous scaffolds), too soft (hydrogels) or too stiff and brittle (films). Additionally, collagen-based biomaterials can be regarded as flexible or bendable but do not exhibit elastic properties, like in many organs. Other biomaterials like elastin can be used or even added to the collagen to modify the mechanical properties of the scaffold. However, the use of elastin can give rise to unwanted side effects like calcification.[16] It is possible that the use of multiple materials in a scaffold with highly different mechanical properties may cause early structural failure of one of the components.[14] Additionally, with regards to the regulatory affairs, restricting the number of ingredients can simplify future applications. Using intelligent design, we set out to find a method to influence the mechanical properties of collagen-based scaffolds using minimal modification. In this manuscript we investigate a method where collagen scaffolds were given elastic-like properties using physical restraint during carbodiimide-based crosslinking.

2. MATERIALS AND METHODS

2.1 TYPE I COLLAGEN FIBRILS

Highly-purified type I collagen fibrils were obtained as previously described.[17] Briefly, bovine achilles tendons were pulverized (0.5 mm) in a universal cutting mill (Pulverisette[19], Fritsch GmbH, Idar-Oberstein, Germany) under liquid nitrogen-cooled conditions. The purification process included washings with aqueous solutions of NaCl, urea, diluted acetic acid, acetone and demineralized water.

2.2 SCAFFOLD CONSTRUCTION

2.2.1 PREPARATION OF A POROUS COLLAGEN SCAFFOLD

A 0.8% (w/v) collagen suspension was prepared by mixing insoluble type I collagen with 0.25 M acetic acid. This suspension was swollen overnight. The suspension was homogenized using a Silverson L5M-A laboratory mixer (Silverson, Chesham, UK). The suspension was mixed for 3 min at 2,500 rpm using the general purpose disintegrating workhead, 3 min at 2,500 rpm using the slotted disintegrating workhead and was subsequently centrifuged at approximately 100 g to remove air. All steps were performed at 4°C to prevent denaturation of the collagen. The collagen suspension was poured into a 10 ml polypropylene mold with a 6 mm stainless steel (grade 304) mandrel inside and frozen for at least 4 h at -20°C in aluminum freezing blocks. Finally, the frozen constructs were lyophilized (Zirbus sublimator 500II, Bad Grund, Germany) after removal of the mandrels.

2.2.2 PREPARATION OF COMPRESSED AND CORRUGATED COLLAGEN SCAFFOLDS

After freeze-drying, the porous constructs (**P-tube**), with the mandrels again included, were uniformly compressed around the mandrel by gently squeezing them between two plane surfaces under a rolling motion to create compressed scaffolds (**C-tube**). Next, to create compressed and corrugated scaffolds (**CC-tube**), two discs fitting exactly over the mandrels, were positioned around each side of the scaffold and subsequently pushed together until 10 mm space was left in between (Figure 1A). The discs were replaced by 4 mm rubber O-rings to hold the scaffold in its corrugated state. Hereafter, the scaffolds were crosslinked using zero-length crosslinker chemistry, N-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC, Merck Schuchardt OHG, Hohenbrunn, Germany) and N-hydroxysuccinimide (NHS, Fluka Chemie AG, Buchs, Switserland) chemistry.[18] Scaffolds were incubated for 3 h at room temperature in 50 mM 2-morpholinoethane sulphonic acid (MES buffer, pH 5.0) (USB, Ohio, USA) containing 40% (v/v) ethanol, 33 mM EDC (Fluka Chemica, AG Buchs, Switzerland) and 6 mM NHS (Fluka Chemica). Two washing steps of 1 h with 0.1 M Na_2HPO_4 (Merck, Darmstadt, Germany) in MilliQ were performed to stop the crosslinking. Next, the scaffolds were washed twice for 1 h in 1 M NaCl in MilliQ, followed by four washing steps with 2 M NaCl (first step 16 h incubation, other steps 30 min). To desalt, scaffolds were washed six times with MilliQ water. Finally, scaffolds were placed in 70% (v/v) ethanol and stored at -20°C until use.

2.3 SCAFFOLD CHARACTERIZATION

2.3.1 MACROSCOPIC EVALUATION AND VIDEO RECORDING

Macroscopic images were taken using a Canon 1d X with a Canon EF macro lens camera (Canon, Melville, NY, USA). High-speed videos (1000 frames per second) were recorded using a Casio EX-ZR100 (Casio, Tokyo, Japan) with camera speed settings at HS1000 and resolution at 224x64 pixels.

2.3.2 SCANNING ELECTRON MICROSCOPY

SEM was used to analyze the morphology and structure of the tubular scaffolds. Samples were lyophilized, fixed on a stub with double-sided carbon tape and sputtered with an ultrathin gold layer in a Polaron E5100 Coating System. Examination was performed in a JEOL SEM 6310 apparatus (JEOL Ltd, Tokyo, Japan) with an accelerating voltage of 10 kV.

2.3.3 MECHANISM ELASTIC-LIKE CHARACTERISTICS

Corrugated scaffolds were air-dried in a stretched position and stayed in this position after drying. Next, scaffolds were placed in solvents with different polarities (i.e. water, ethylene glycol, methanol, ethanol, 1-propanol, 1-butanol, acetone and chloroform, all Sigma-Aldrich). Before use, each solvent was dehydrated by anhydrous $Cu(II)SO_4$ crystals (Sigma-Aldrich). The degree of corrugation in each solvent was measured as a percentage of the maximal corrugation (water was set to 100%). This experiment was performed three times independently.

To visualize the effect of the polarity of the solvent on the degree of corrugation a two-layered experimental setup with water and chloroform was designed. An air-dried stretched scaffold was placed in a tube and chloroform was added until half of the scaffold was submerged. Next, water with $Cu(II)SO_4$ (to increase the contrast between the two layers) was added on top of the chloroform. Images were taken every hour (Sony Cyber-shot DSC-H10).

2.3.4 MECHANICAL PROPERTIES

To characterize the mechanical properties of the scaffolds, force-monitored ultimate tensile strength experiments were performed and force-distance curves were determined by using a Zwick/Roell Z2.5 testing machine (Zwick/Roell, Ulm, Germany). From this curve ultimate tensile strength, work needed for break and stiffness were derived. Tests were performed by clamping 2 mm of the scaffolds between two custom made clamps (supplementary figure 1) and pulling them apart in opposite direction with 50 mm/min (n=19). For P-Tube and C-Tube scaffolds, force monitoring

started when scaffolds transitioned from their relaxed state (original length after crosslinking).

For fatigue tests, corrugated scaffolds, which were continuously wetted with phosphate buffered saline using a peristaltic pump, were extended 1,000 times with 167 mm/min to reach a force of 0.4 N whilst monitoring the distance (n=4). Data was processed using TestXpert II V3.5 software (Zwick/Roell).

2.3.5 PRIMARY AMINE GROUPS

The degree of crosslinking of P-Tube, C-Tube and CC-Tube was evaluated by the loss of primary amine groups upon crosslinking as measured by a reaction with trinitrobenzene sulfonic acid.[19] In brief, freeze-dried samples were incubated for 30 min in 4% (w/v) $NaHPO_4$ (Merck, Darmstadt, Germany) at room temperature. Next, 0.5% (w/v) 2,4,6-trinitrobenzesulfonic acid (Fluka Chemie AG, Buchs, Switzerland) was added and incubated for 2 h at 40°C. The samples were hydrolyzed with 6 M hydrogen chloride for 1.5 h at 60 °C. MilliQ was added to dilute the samples before they were measured at 420 nm with a spectrophotometer (Bio-Tek, Bad Friedrichshall, Germany). Glycine (Scharlau Chemie, Barcelona, Spain) was used for the calibration curve. Three different scaffolds (for P-Tube, C-Tube and CC-Tube) in triplicate were analyzed in three different TNBS assays (with 9 samples per condition) and compared to non-crosslinked samples (3 samples).

2.4 *IN VITRO* EVALUATION

2.4.1 CELL CULTURE

NIH/3T3 fibroblasts (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's Medium Glutamax (Life Technologies, Carlsbad, CA, USA) enriched with 10% fetal bovine serum (GE Healthcare, PAA Laboratories, Pasching, Austria), penicillin (1,000 I.U./ml) and streptomycin (1,000 µg/ml) (PAA Laboratories) at 37°C under an atmosphere containing 5% CO_2. Confluent cells were harvested using 0.25% (w/v) trypsin-EDTA (Life Technologies).

2.4.2. CELL SEEDING ON SCAFFOLDS

For the cell seeding experiment, scaffolds were cut into strips of 0.5 x 2 cm (P-Tube and C-Tube), and 0.5 cm by 1 cm for CC-Tube. The strips were disinfected by six washings steps with 70% (v/v) ethanol of which one was an overnight step. To remove ethanol, six washing with 0.1 M sterile phosphate buffered saline (pH 7,4) (PBS, Braun, Melsungen, Germany) were performed with one overnight step. Finally, strips were incubated in culture medium for 1 h prior to cell seeding. The strips were transferred to a 6-well suspension plate and 450,000 cells in 750 µl were added to each scaffold. After three hours, 3,250 µL of culture media was added. Constructs were cultured for four days and media was renewed every other day.

2.4.3 ALAMAR BLUE CELL VIABILITY ASSAY

To investigate the cell viability of the 3T3 fibroblasts seeded on the scaffold, an Alamar Blue (Life Technologies, Carlsbad, CA, USA) assay was performed. For each scaffold type 6 mm punches were taken and subsequently placed in 96-wells plates. Next, 10,000 cells in 25 µL were seeded in each well in triplicate and after 3 h 200 µL culture media was added. Alamar Blue was added 4, 24 and 96 hours after seeding. Fluorescence was measured using an excitation wavelength of 540 nm and an emission wavelength 620 nm. This experiment was performed three times independently.

2.4.4 HISTOLOGICAL EVALUATION OF SCAFFOLDS

For histology the constructs were placed in Tissue-Tek (Sakura, Torrance, USA) and frozen in dry-ice cooled 2-methylbutane (Sigma-Aldrich, St. Louis, USA). Next, they were sectioned (5 µm) using a cryostat microtome (Heidelberg, Heidelberg, Germany), mounted on superfrost slides (Thermo Scientific, Menzel GmbH & Co KG, Braunschweig, Germany) and stored at -20 °C until use. Before staining, the slides were fixed using cold acetone (-20 °C) for 15 min. Next, the slides were stained with hematoxylin and eosin (HE). Slides were analyzed using a Leica CTR6000 microscope (Leica Microsystems GmbH, Wetzlar, Germany).

3. RESULTS

3.1 SCAFFOLD MORPHOLOGY AND DEGREE OF CROSSLINKING

The swelling, homogenization, freezing and freeze-drying of bovine collagen type I resulted in porous tubular scaffolds, which were comparable to scaffolds previously described in literature (Figure 1B-D).[20, 21] The freeze-dried porous tubular scaffold (P-Tube) was used as a starting point to produce a collagen scaffold with elastic-like characteristics using two steps. Compressing the porous scaffold around the mandrel resulted in a dense film-like scaffold with a lower porosity (C-Tube, Figure 1E-G). Corrugation of the C-tube scaffold resulted in a tubular scaffold with low porosity and a pleated/corrugated wall structure (CC-Tube, Figure 1H-J). The chemical crosslinking of the scaffolds resulted in a fixation of either the porous or dense collagen structure, with or without the corrugated morphology. The degree of the crosslinking was determined by measuring the content of free amine groups. Non-crosslinked collagen showed an average of 247±15 nmol of free amine groups per mg collagen. After crosslinking P-Tube, C-Tube and CC-Tube had averages of 125±17, 91±14 and 119±16 nmol free amine groups per mg collagen, equating approximately to a reduction of 50, 63 and 52% of amine groups, respectively (Table 1).

TABLE 1. DEGREE OF CROSSLINKING

Sample	Average		S.D.	Free Anime Reduction (%)	Sample Size
	NH_2 (nmol)/Collagen (mg)				
Non-Crosslinked Collagen	247	±	15	0	3x3
Crosslinked P-Tube	125	±	17	50	3x9
Crosslinked C-Tube	91	±	14	63	3x10
Crosslinked CC-Tube	119	±	16	52	3x11

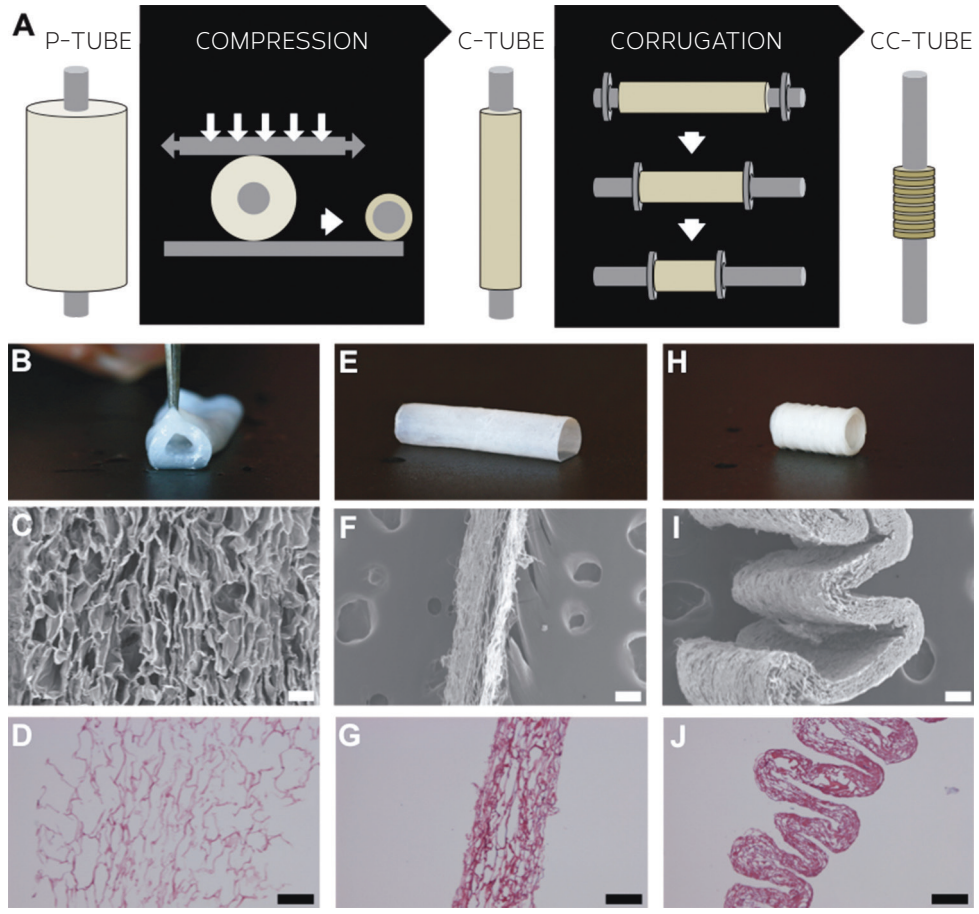

FIGURE 1: SCAFFOLD PRODUCTION METHOD AND MORPHOLOGICAL CHARACTERISTICS.

A) Schematic representation of the preparation method of P-tube, C-tube and CC-tube using compression and corrugation. **B–D)** Macroscopic image, scanning electron micrograph and H&E staining of P-Tube, where the porous morphology is clearly seen. **E–G)** Macroscopic image, scanning electron micrograph and H&E staining of C-Tube, where the dense collagen structure as a result of the compression is shown. **H–J)** Macroscopic image, scanning electron micrograph and H&E staining of CC-Tube, where the induced corrugated structure of the scaffold wall is apparent. Scale bars SEM = 100 μm. Scale bars H&E = 200 μm.

3.2. MECHANICAL CHARACTERISTICS

The previously mentioned scaffolds were subjected to mechanical characterization. The CC-Tube scaffolds displayed elastic-like behavior, where the scaffold could be elongated to nearly the length before corrugation (C-Tube) and returned to its relaxed position upon release of the force (Figure 2 A-C). The initial elongation/unfolding of the CC-Tube scaffolds seemed to cost little effort as the corrugated wall structure is merely unfolding and the collagen is only exposed to elastic deformation. Eventually a sharp increase in force is observed during the elongation, indicating that the corrugated structure is completely unfolded (plastic deformation). To quantify the differences between the different scaffold types, the mechanical properties of the total tubes were tested using a custom-made clamp, which could hold the entire tubular scaffold during a tensile test. During tensile testing, the three scaffolds displayed entirely different mechanical properties as seen by the shape of the curves. Indeed the CC-Tube scaffold did not need large amounts of force (0.1 N) in order to be extended up to about 3.4±0.2 cm. After this point, the CC-Tube behaved like an intermediate of P-Tube and C-Tube. A representative curve from each scaffold type is shown in Figure 2D. CC-Tube could be elongated and stretched the furthest until rupture (5.8 ± 0.5 cm), followed by P-Tube (1.8 ± 0.3 cm) and C-tube (1.2 ± 0.2 cm) (Figure 2E). This coincides with the results of the scaffold stiffness, where C-Tube was significantly stiffer (4.0 ± 0.5 N/mm) than P-Tube (1.6 ± 0.4 N/mm) and CC-Tube (1.6 ± 0.3 N/mm) where P-Tube and CC-Tube did not differ in degree of stiffness (Figure 2F). C-Tube was significantly stronger (4.2 ± 1.1 N) than CC-Tube (3.4 ± 1.0 N) and P-Tube (1.8 ± 0.5N), respectively (Figure 2G). Work needed for break (or area under the curve) showed that CC-Tube required significantly more work until scaffold failure (36 ± 8.6 J) than C-Tube (24 ± 9.2 J) and P-Tube (16 ± 5.7 J) (Figure 2H). The elastic-like behavior of the CC-Tube was tested using a fatigue test where the CC-Tube scaffolds were subjected to 1000 cycles of elongation up to a fixed force (4 N) whilst recording the force (Figure 2I). Averages and standard deviations of the peak values were plotted, where the curves show a steep initial decline of tensile force from approximately 0.4 N to 0.37 N in the first 200 cycles, corresponding to a decline of 1.5×10^{-4} N/cycle. In the following 800 cycles, the approximate rate of decline was only 3.0×10^{-5} N/cycle (0.37 N to 0.346 N). The repeated unfolding and refolding of the corrugated structure show convincing

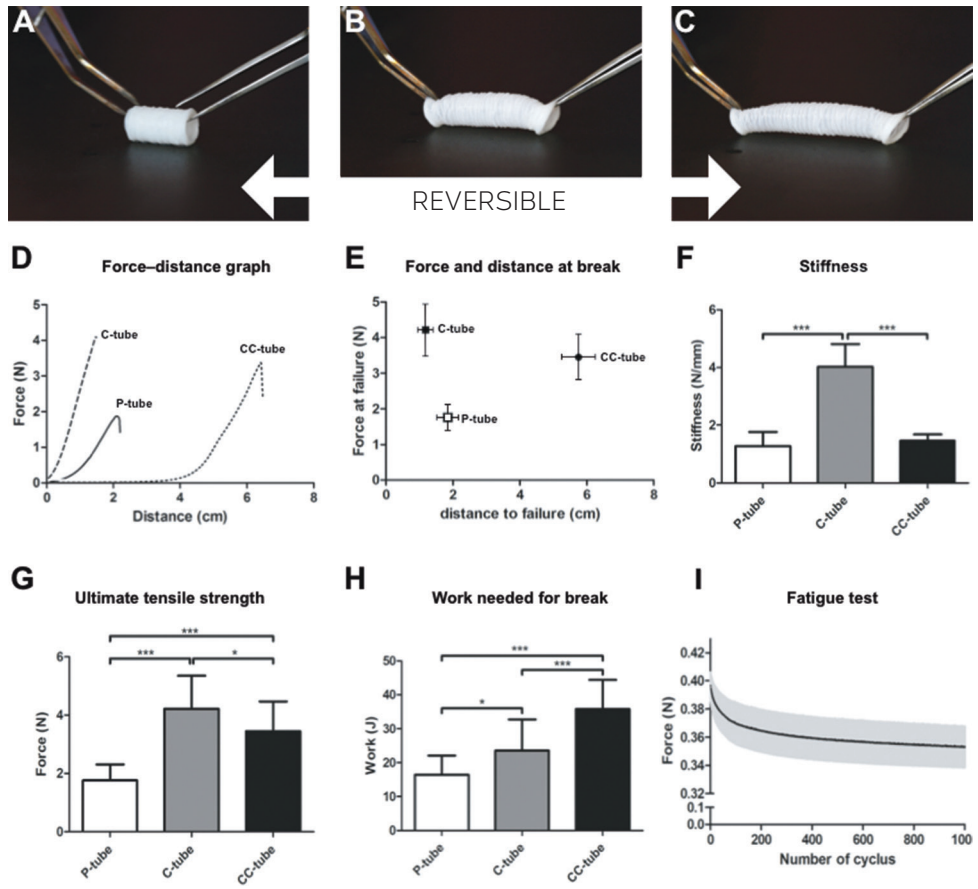

FIGURE 2: MECHANICAL CHARACTERIZATION OF P-TUBE, C-TUBE AND CC-TUBE SCAFFOLDS.

A) CC-Tube in relaxed position. **B)** CC-Tube in semi-extended position. **C)** CC-tube in fully extended position. **D)** Representative force-distance graph of P-tube, C-tube, CC-tube, where the differences in extendibility are depicted. **E)** Displacement and force at failure for P-tube, C-tube and CC-tube (n=19 per scaffold type) showing the range at which the scaffolds fail. **F-H)** Stiffness, ultimate tensile strength and work for each scaffold type derived from force distance graph (n=19); One-way ANOVA with Bonferroni post-hoc test * = P > 0.05, ** P > 0.01, *** P > 0.001. **I)** Fatigue test where CC-tube was monitored during repeated elongation cycles 1000 cycles (n=4). The graph demonstrates that the folding and unfolding of the pleated structure only shows a slight decrease in mechanical integrity.

evidence that the unfolding and refolding of the corrugated structure holds for at least 1000 cycles.

3.3 ELASTIC-LIKE MECHANISM

The mechanism behind the elastic-like behavior was investigated using liquids with different relative polarities. When the CC-Tube was air-dried in a stretched state, it would remain in this position. Upon rehydration of a dried CC-Tube scaffold in water, it would return to its original length (Figure 3A). Other liquids with lower polarities were tested in this scenario and a trend was seen where a decrease in relative polarity hampered the return of the scaffold to its corrugated state (Figure 3A and B). To further elucidate this phenomenon, a test was designed where the dried CC-Tube was placed in a test-tube with liquids on both ends of the polarity scale (water and chloroform). Since the liquids are immiscible, they formed a sharp barrier. To emphasize the separation between the two different layers, the water was colored with $Cu(II)SO_4$.[22] The scaffold was placed in the chloroform layer after which the water was added on top. The part of the CC-Tube scaffold protruding in the water layer returned to the corrugated state, whereas the CC-Tube segment in the chloroform remained stretched. Over time, the CC-Tube scaffold slowly moved towards the water layer indicating an innate affinity for water (Figure 3C).

3.4 *IN VITRO* EVALUATION

The results of the Alamar Blue experiment showed that after compression and corrugation the scaffolds are still cytocompatible (Figure 4D). After 24 and 96 h, an increased fluorescent value was measured corresponding to an increased number of cells. Histological evaluation (Figure 4A-C) confirmed the outcome of the cell viability assay, however they also revealed that cells did not penetrate the dense collagen structure of the C-Tube and CC-Tube. The cells appeared to form large sheets on top of the scaffold resulting locally in a higher cell density.

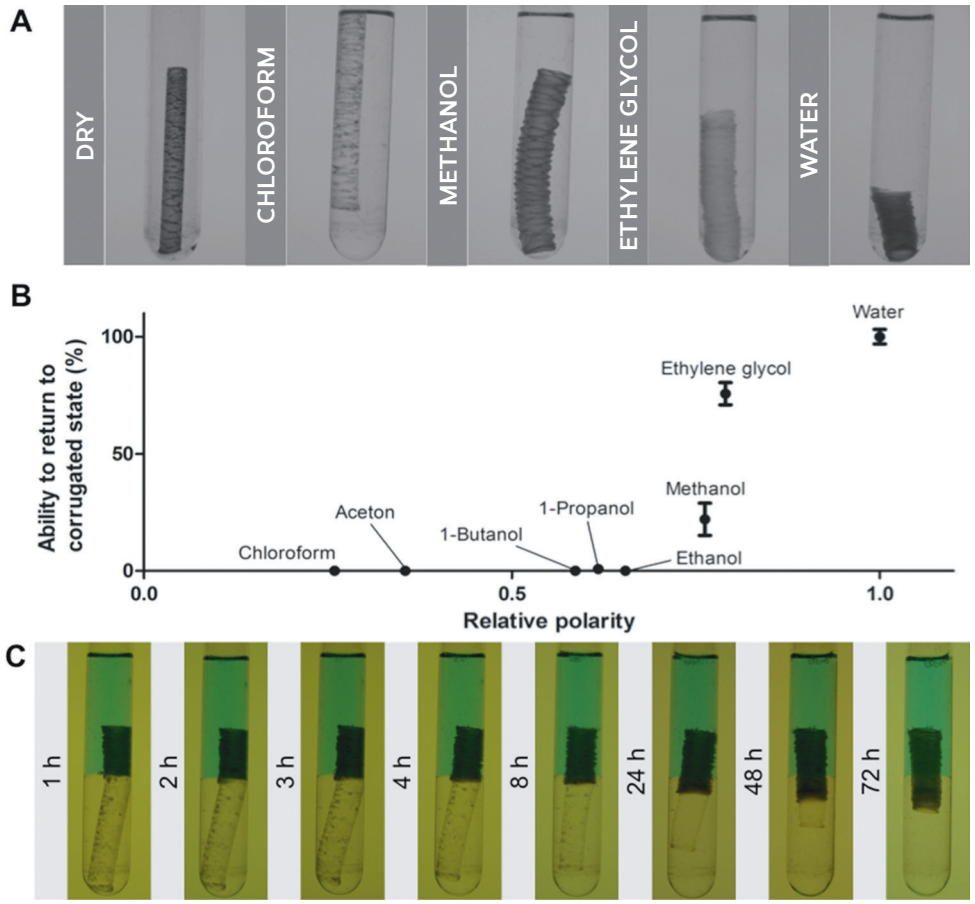

FIGURE 3: MECHANISM BEHIND ELASTIC-LIKE BEHAVIOR.

A) Air-dried CC-Tube exposed to different solvents, depicting the stretched state in which the air-dried scaffolds stay and subsequently the length to which they return after various solvent are added. **B)** Ability of air-dried CC-Tube to return to the corrugated state in different solvents (n=3). **C)** Time-lapse of an air-dried CC-Tube in a two component chloroform (bottom half, colorless) and water (top half, light blue) system, showing a gradual return of the scaffold in its stretched state to the hydrated and subsequently corrugated state.

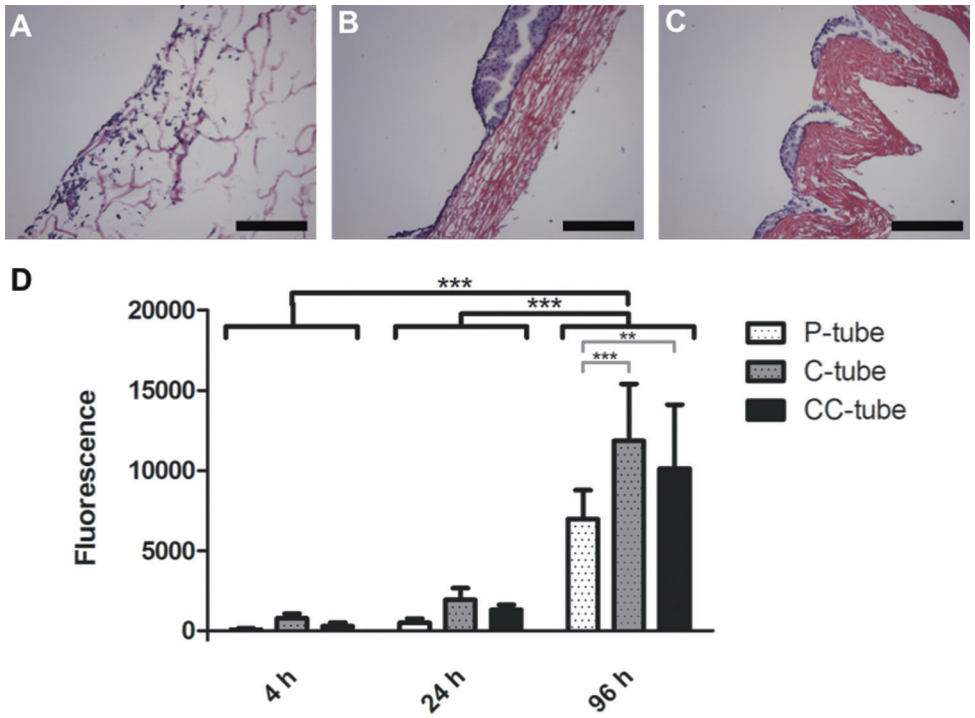

FIGURE 4: *IN VITRO* CYTOCOMPATIBILITY.

A) Microscopical image of HE stained cross-section of P-Tube after culturing with fibroblasts for 4 days. **B)** Microscopical image of HE stained cross-section of C-Tube. **C)** Microscopical image of HE stained cross-section of CC-Tube. All bars 200 µm. **D)** Alamar blue cell viability assay at 4, 24 and 96 hours where a one-way ANOVA with Bonferroni post-hoc test was performed. Differences within time point at 96 h were analyzed using a two-way ANOVA with Bonferroni post-hoc test. * = P > 0.05, ** P > 0.01, *** P > 0.001.

4. DISCUSSION

4.1 SCAFFOLD CHARACTERISTICS

Methods to alter the mechanical properties of porous collagen scaffolds have been extensively studied in literature.[23-26] Studies have focused on increasing the overall strength by using either chemical modification or the addition of other components such as synthetic polymers.[24, 27] Dense collagen films made by compressing porous collagen scaffolds have been previously described in literature, however, were mainly used as a strengthening component.[21, 28] In this manuscript we have described a method wherein elastic-like properties can be introduced to collagen-only scaffolds using a combination of chemical crosslinking and physical restraint.

4.2 ELASTIC-LIKE MECHANISM

The unfolding and refolding of the corrugated structure seems to be a form of macrostructure induced elasticity. The total scaffold appears to have elastic-like properties where the scaffold can be elongated and will return to its relaxed state, however, the collagen fibers itself do not display elastic properties, especially in comparison to elastin.[29, 30] The mechanical properties of corrugated collagen fibers in native artery tissue has been previously described, where the morphology allows for circumferential expansion of the arteries to encompass the pulsing blood and pressure from surrounding tissue.[31] However, the mechanism behind the phenomenon of an elasticized collagen scaffold (CC-Tube) has, to the best of our knowledge, not been described in previous literature for collagen-based materials.

Several dedicated experiments were designed to elucidate the mechanism behind the elastic-like properties. First of all, the scaffold did not exhibit elastic-like properties when it was dried, in both relaxed and stretched state. If the scaffold were to be rehydrated, it would return to its relaxed state. This led us to believe that an aqueous environment was of utmost importance for the corrugated scaffold to exhibit elastic-like properties. When the stretch-dried corrugated collagen scaffolds were exposed to solvents of varying polarity, liquids/solvents with a relative polarity below

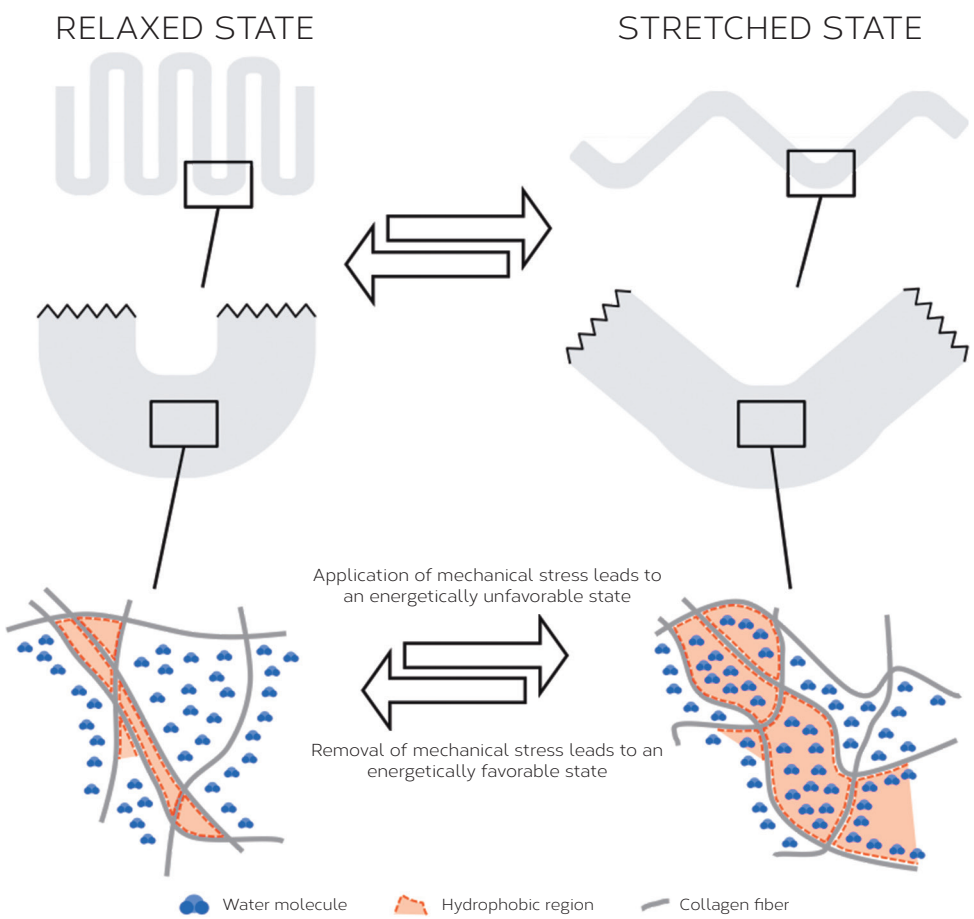

FIGURE 5: HYPOTHESIZED MOLECULAR MECHANISM BEHIND ELASTIC-LIKE PROPERTIES.

Schematic representation of molecular mechanism behind the elastic-like properties of CC-Tube. The scaffold in its relaxed state (left) may have areas that are hydrophobic and consequently contain little water. When an external force is applied to the scaffold that causes it to extend (right), water could enter these hydrophobic regions. However, the water may exert some form of hydrophobicity induced pressure on the collagen structure which forces the return to its relaxed state once the external force is removed.

0.65, e.g. chloroform, acetone or ethanol, did not cause the dry scaffold to return to its corrugated state. This indicates that hydrophobic interactions play an important role in the elastic-like behavior. Evidence leaves room for speculation, where the collagen scaffold might prefer an aqueous/polar environment to nonpolar solvents. This notion coincides with the two-component (water and chloroform) test.

Taking all the evidence into account, the possible driving mechanism behind the elastic-like properties of collagen is presented in Figure 5. Based on the evidence, we hypothesize that due to the compression and subsequent corrugation before and during carbodiimide crosslinking, certain hydrophobic regions are created where the collagen is densely packed together. In non-crosslinked collagen, amine and carboxylic groups exist as free, charged, groups and add to the overall hydrophilicity of the collagen. Once these groups are involved in the formation of a peptide bond, a much less hydrophilic moiety, the overall hydrophobicity increases. We hypothesize that the crosslinks are formed in such a way that the overall energy of the scaffold is lowest in its corrugated state. It may be possible that due to compression and corrugation, small density differences are induced which after crosslinking may result in regions with higher or lower hydrophobicity. We currently cannot explain what exactly causes the differences in hydrophobicity, but causes may include; **1)** the physical location or positioning of the crosslink or **2)** an increase in amine and carboxylic groups which are sterically able to form crosslinks, which subsequently increases the overall number of crosslinks, ergo, the overall hydrophobicity of that region.

We further hypothesize that, once energy in the form of tensile force is used to disrupt the thermodynamically favorable conformation of the corrugated scaffold in its relaxed state, water can enter the dense hydrophobic regions in the collagen, which is thermodynamically unfavourable. Once the externally applied tensile force is removed, the hydrophobic regions will return to their original, hydrophobic, molecular environment, resulting in refolding of structure. This proposed mechanism is strikingly similar to the molecular mechanism behind the elasticity of another ECM protein, elastin.[32-34] The exact modus of action may still be unclear but we firmly believe that the difference in hydrophobicity of the different regions in the corrugated scaffold may play in important role in the unfolding and refolding of the corrugated morphology.

4.3 CYTOCOMPATIBILITY

Collagen is a highly characterized biomaterial and the cellular response to collagen-based biomaterials is generally well-understood.[35-37] However, modification of the collagen structure or chemical make-up can influence the cellular response.[38] In the case of the compressed (C-Tube) and corrugated (CC-Tube) scaffolds, the effect of compression, corrugation and subsequent crosslinking was unknown. In this respect it was necessary to determine the biocompatibility of the constructs after the processing steps. In the low porous C-Tube and CC-Tube cells did not penetrate the scaffold and appear to form sheet-like structure on top of the scaffold. After 96 h of culturing, both C-Tube and CC-Tube scaffolds seem to contain an equal or higher amount of viable cells compared to P-Tube scaffolds. This difference may be caused by the decreased porosity, ergo a decreased surface area, after compression. Consequently, the initial cell density after seeding was higher in C-Tube and CC-Tube scaffold, therefore leading to increased cell-cell interactions which may be favorable for cell viability.[39]

The effect of crosslinking has been previously studied *in vitro* and *in vivo*, where, depending on the type of crosslinker, the results greatly vary.[40, 41] Cytocompatibility is generally not influenced by the crosslinking, however remnants of the crosslinker itself may have a cytotoxic effect.[42, 43] The method of crosslinking may also change the physiochemical and mechanical properties such as hydrophobicity and stiffness, which may in turn be detrimental to the *in vitro* cell growth. Additionally, the use of zero-length crosslinking methods, in this case carbodiimides, does not introduce moieties foreign to the human body, as frequently is the case with aldehyde-based agents.[44] As the biocompatibility of the P-Tube has been extensively studied in both *in vitro* and *in vivo* studies, it is a suitable benchmark for the C-Tube and CC-Tube.[45-48] Overall, the cytocompatibility of the C-Tube and CC-Tube seems to be comparable to the P-Tube. This indicates that the compression, corrugation and subsequent crosslinking do not negatively affect the intrinsic biocompatibility of collagen.

4.4 IMPLICATIONS AND APPLICATIONS

To the best of our knowledge, only decellularization-based methodologies have been able to produce scaffolds which may have a pleated structure.[49-51] Other methods to induce elasticity to collagen-based scaffolds mainly involve the addition of other components, like (recombinant or natural) elastin, (recombinant and natural) silk and synthetic polymers.[5, 52] Additionally, elastin-based biomaterials applied *in vivo*, have shown to induce unwanted side effects like calcification. Using elasticized collagen-only materials may circumvent the use of elastin. Nevertheless, despite some clinical successes, the mechanical compliance of acellular collagen-based materials can still be dissimilar to the target tissue. For example, some tissues may undergo repeated elastic deformation like in the unfolding of pleated ECM structures of e.g., the skin, urinary and gastrointestinal tract.[49, 53, 54] These structures, like in the corrugated scaffolds, can also unfold to some extent without an initial increase in strain.[55] By compression, corrugation and subsequent crosslinking, the collagen scaffolds were given a corrugated wall structure, which in some ways resemble these pleated structures. Collagen-based biomaterials currently under investigation for use in repairing the aforementioned tissues could also be altered using the methodology presented in this manuscript. In this way, not only the mechanical properties but also the morphological characteristics may be fine-tuned to the specific application. Despite the corrugated morphology of the presented CC-Tube, other applications might require unfolding/elasticity in a different direction to encompass luminal expansion or peristaltic movements (like in the intestines, esophagus, urethra or ureters).[56] Efforts are being made to induce a elasticized star-shaped lumen in a CC-Tube scaffold that can be used for previously mentioned tissues.

5. CONCLUSION

Collagen is generally known for providing strength and rigidity to tissues, but not for providing elasticity. To the best of our knowledge, literature has never described a method where elastic-like properties are induced to a collagen-only scaffold. For tissue engineering and Regenerative Medicine purposes, the methodology described in this manuscript may be a suitable tool to reduce the dissimilarities in mechanical characteristics between the scaffold and the target tissue.

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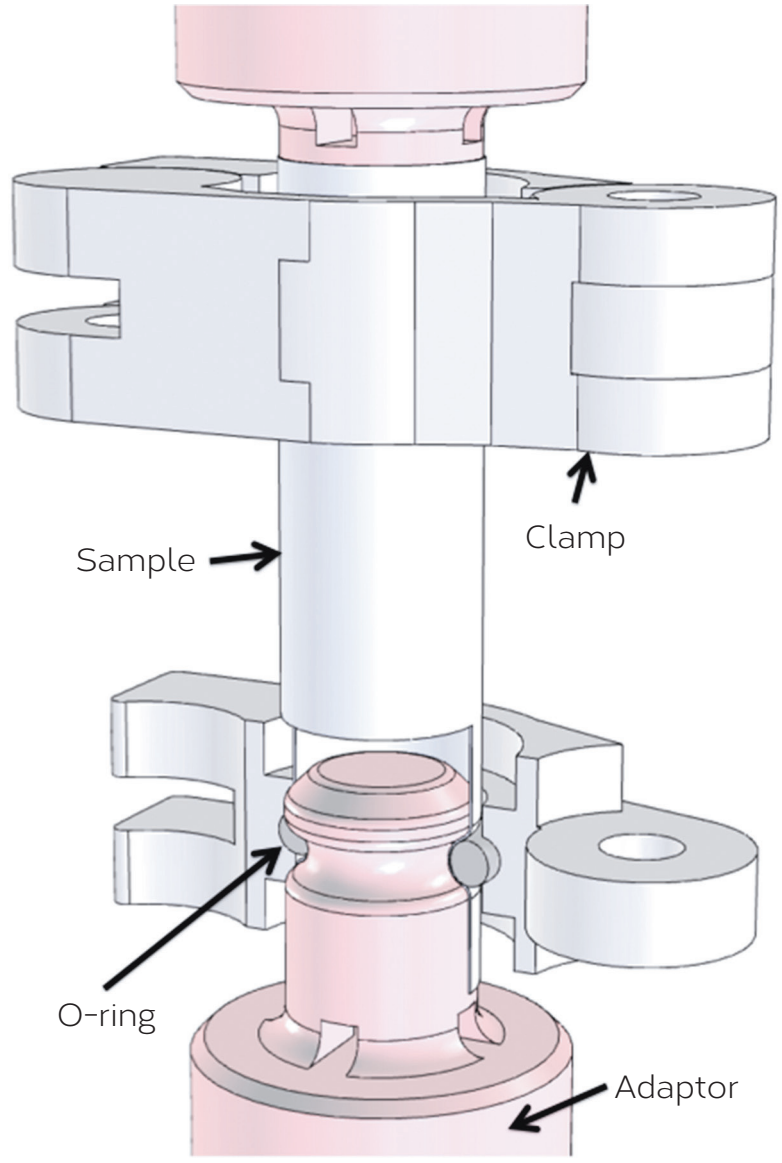
LIST OF FIGURES & TABLES



SUPPLIMENTARY FIGURE 1: SCHEMATIC OVERVIEW OF THE CUSTOM-MADE CLAMP FOR TUBULAR SCAFFOLDS.

To allow for the mechanical evaluation of the scaffolds in tubular form a custom-made clamp was made to fit the Zwick/Roell device. The adaptor cylindrical shape could be inserted into a tubular scaffold after which a clamp fitted with a rubber ring could be used to secure the sample in place. Several openings were added to the design of the adaptor to allow for free flow of air during repeated stretch and relaxation cycles.

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CHAPTER 08

SUMMARY, GENERAL CONCLUSION AND FUTURE PERSPECTIVES

SAMENVATTING, ALGEMENE CONCLUSIE EN TOEKOMSTVISIE

SUMMARY

Regenerative Medicine is a new rapidly growing discipline in medical science.[1] One of the most important strategies in Regenerative Medicine is based on stimulating the body's natural healing mechanism by implanting materials which mimic the extracellular matrix (ECM), with or without cells, into a defect.[2] The ECM essentially acts as a template in the early stage of regeneration for cells to adhere or migrate to, proliferate and differentiate. Ideally, this hinders the formation of scar tissue and subsequently stimulates formation of new functional tissue, thus regenerating the tissue/organ.[3] Materials mimicking the ECM, also referred to as biomatrices or scaffolds, can be classified into two groups: the decellularized tissues[4, 5] or molecularly defined constructs comprised of natural and/or synthetic biomaterials.[5, 6] Depending on the target tissue/organ, the scaffold needs to meet different biological and mechanical requirements.

In **chapter 1** an extensive overview is presented of the different strategies, materials and methodologies used in tissue engineering and Regenerative Medicine. Subsequently in **chapter 2** the current status of tissue engineering and Regenerative Medicine of the respiratory system, including the lung, tracheobronchial system and the diaphragm, is reviewed. By scrutinizing the latest literature, it is concluded that, despite many promising proof of concepts, still a long road lies ahead with many foreseeable and unforeseeable pitfalls.

Generally, the tissue engineering and regenerative medicine applications, which are most within reach, depend on the 'simplicity' of the target tissue. The reconstruction of tissues with functions that are primarily of mechanical nature seems to follow a more straightforward approach. As with many treatment modalities, the success of a new technique often lies in its simplicity.[7] Target tissues with primarily mechanical functions obviously include bone, cartilage and ligaments. However hollow organs such as the trachea, larger blood vessels, esophagus and the lower urinary tract also have mechanical functions.[8] The aim of this thesis was to develop new techniques and modify existing procedures to contribute to the currently available scaffold armamentarium for repairing defects to large hollow organs.

POROUS COLLAGEN-BASED TUBULAR SCAFFOLDS

Porous scaffolds can be constructed by homogenizing highly purified acid-swollen insoluble bovine type I collagen (hereafter referred to as collagen) followed by controlled freezing and subsequent freeze-drying.[9] This process yields a micro-porous collagen scaffold that has been successfully used as an ECM-mimicking material to stimulate regeneration of tissue segments *in vivo*.[10, 11] Changing the mold, in which the casting and freezing process is conducted, can produce tubular variants of the scaffolds.[12] Up until now the diameter investigated was generally limited to small diameter applications like blood vessels and lower urinary tract.[13] For larger diameter applications (>ø15 mm), the process was revisited and optimized.

In **chapter 3**, this process is described where large diameter tubular collagen-based scaffolds were designed for possible future application in the regeneration of the esophagus.[14] Additionally, to encompass the increase in size, an attempt was made to modify the mechanical properties by the addition of either an additional collagen layer or a synthetic polymer knitting (polycaprolactone). Since the lack of suitable vasculature in larger constructs often leads to failure of the treatment modality, the previously mentioned scaffolds were subjected to omental wrapping in an ovine model as an attempt to pre-vascularize the constructs. Immunohistochemical evaluation after 6 weeks of incubation revealed newly formed vasculature entirely throughout the collagen-based scaffolds. Moreover, signs of inflammation or calcification were largely absent. This indicates that large diameter tubular scaffolds in combination with omental pre-implantation might prove to be a valuable addition to the tissue engineering and Regenerative Medicine toolbox.

The development of large diameter collagen-based scaffolds was continued in **chapter 4**, were the process was once again revisited to be able to produce a collagen - polymer hybrid scaffold with a considerable length for use as an urinary diversion.[15] A commercially available medical-grade polymer knitting (VYPRO II Mesh) was incorporated in a tubular collagen scaffold with a length of 12 cm, luminal diameter

of 15 mm and wall thickness of 6 mm. The scaffolds were sterilized and implanted as an incontinent urostomy using the right ureter in a porcine model. Results were encouraging, but not entirely positive. In most cases, the scaffold successfully functioned as an incontinent urinary conduit, showing neo-vascularization of the construct and re-urothelialization of the lumen. However, the semi-absorbable synthetic mesh (combination of VICRYL and PROLENE) seemed to elicit unwanted biological responses. Fibroblast deposition was observed near the polymer knittings and subsequently induced tissue contraction. In the **chapters 3 and 4**, the potential of a collagen-polymer hybrid scaffold was demonstrated and simultaneously the importance of choosing the correct polymer type for each application was stipulated.

The addition of other natural or synthetic polymers is a logical method, which is frequently chosen to modify the mechanical properties of a collagen-based scaffold. However, methods based on modifying the properties of collagen itself are limited. Carbodiimide crosslinking is a popular technique to strengthen collagen scaffolds. Despite its ubiquitous in combination with collagen, the full potential of carbodiimide crosslinking has yet to be realized. In **chapters 3 and 6** a novel method is described which expands the number of options to alter the mechanical properties of collagen. By physically restraining porous collagen scaffolds during carbodiimide crosslinking in different manners, the mechanical properties of the scaffold can be modified to become more rigid, stiff or elastic. In **chapter 6**, the wall of a porous tubular scaffold was compressed to approximately 10% of its original thickness and subsequently corrugated before carbodiimide crosslinking. This process resulted in a collagen scaffold with elastic-like properties. The wall compression alone caused an increase in density and ensuing increase of scaffold stiffness. The corrugation caused the scaffold wall to form micro-folds, which were fixated during crosslinking. The folded or corrugated structure of the collagen wall allows it to be unfolded without causing damage to the wall. If the unfolding force is removed, the corrugated scaffold returns to its original position. This elastic-like behavior has never been observed in collagen-based scaffolds before. It was thus demonstrated that a combination of physical restraint and crosslinking could induce novel mechanical properties.

NOVEL TECHNIQUES TO PRODUCE LARGE HOLLOW SCAFFOLDS

To mimic larger hollow organs like the bladder, adjustments to the current freezing and freeze-drying techniques were made. **Chapter 7** describes the development of a spherical hollow bladder-shaped scaffold for the future development of a neo-bladder. A custom-made mold was designed to mimic the size and shape of a bladder. Additionally the mold was designed to seamlessly integrate anastomosis sites for the ureters and urethra. Subsequently, a technique was designed where the scaffold could be cast in one step which, after freeze-drying, yielded a hollow porous scaffold. The morphological properties of the scaffold were characterized and showed a closed lumen and unidirectional pore structures directed from the outside towards the center of the sphere. The cytocompatibility was also tested by cultivation of primary porcine urothelial and smooth muscle cells on to the scaffold. Cells attached and proliferated on the bladder scaffold and retained their phenotype as determined by immunohistochemistry. The developed technique to make reproducible seamless hollow collagen-based scaffolds could be the first step to an off-the-shelf product for neo-bladder engineering.

Methods to produce hollow scaffolds are mainly based on the previously mentioned freezing and freeze-drying regimes. In **chapter 5** a novel method was described where tubular scaffolds with controlled collagen fiber alignment were extruded using a counter-rotating cone extruder.[16] A highly viscous collagen suspension (4% w/v) was extruded at a constant speed onto a 25 mm mandrel. During extrusion, a counter rotating force was applied to the collagen. Using multi-photon microscopy, the collagen fibers could be visualized in 3D without any other staining or processing techniques. A clear correlation was found whereby the direction and extent of collagen fiber alignment during extrusion were a function of the shear forces caused by a combination of the cone rotation and flow direction. A gradual change in fiber direction was observed throughout the sections of the scaffold. This novel technology is highly adjustable, controllably produces tubular collagen scaffolds and can be scaled up with ease.

GENERAL CONCLUSION

Insoluble type I collagen is a highly versatile biomaterial compatible with an array of methods for fabrication of extracellular matrices. In this thesis, the limits of collagen-based scaffolds were extended through methods to modify the strength by the addition of a synthetic polymer, to induce elastic-like properties using crosslinking, to modify fiber architecture using shear forces and to make seamless large hollow spheres using an elegant one-step casting technique. The modification of collagen-based scaffolds may play an important role in the fine-tuning of scaffolds for many different treatment modalities of defects to hollow organs.

FUTURE DIRECTIONS

Collagen has been and will most likely remain the 'prince' of the biomaterials. The ubiquitous occurrence of collagen throughout the ECM makes it an ideal candidate for all sorts of tissue engineering and Regenerative Medicine strategies.[17, 18] From the work presented in this thesis we feel that it is clear that collagen should be regarded as the base for many strategies. A very comprehensive overview was recently published where the current status of esophageal Regenerative Medicine was reviewed.[19] As with many tissue engineering and Regenerative Medicine overviews, the authors concluded that more research is necessary. However, the use of collagen in many listed attempts and strategies was not surprising, subsequently supporting our beliefs of the importance of collagen in the success of future strategies.

The biocompatibility of collagen is unrivaled within the category of natural biomaterials. However, difficulty regarding components derived from animal sources is that the derivatives of animal tissues may contain transmissible pathogens such as viruses, bacteria and prions. Currently, most medical-grade collagen is derived from bovine, porcine and ovine origin and is sourced from animals raised in a closed herd setting.[20] Despite the substantial amount of collagen being produced as a by-product of the meat industry, it may very well prove to be difficult to meet the growing demand for certified medical-grade collagen from a controlled ungulate source.

Simultaneously, the isolation of other ECM components from standardized animal sources should be investigated. More effort should be put in finding other molecularly-defined components or modification methods which, with collagen, have a synergistic effect in wound healing and tissue regeneration. It is probable that each target application requires its own 'recipe' or unique combination of collagen with other ECM-based components. Such components can include; glycosaminoglycans, elastin, components of the basement membrane and growth factors.[21] We feel that that the availability of more medical-grade ECM components could lead to an increase in devoted research. Currently, colleague research groups often linger in the development phase, seemingly due to the lack of medical-grade certification for the ECM-based biomaterials. If different research groups work together using standardized commercially available medical-grade materials, the (pre)clinical research barrier may be easier to breach.

Despite the sheer amount of work remaining to be done on finding effective biomaterials and treatment modalities (both acellular and cell-based), the current Regenerative Medicine market is annually worth billions (US$).[22] The Regenerative Medicine community has hopefully learned from the mistakes made during the 1980/90s gene therapy hype; only now, decades later, the first approved gene therapy treatments are starting to become available.[23] It is normal for a new technology to undergo the "Gartner hype cycle", where a promising breakthrough leads to overblown prospects and promises until it becomes clear that significantly more time, money and labor is needed. As the public is letdown, the difficulty in obtaining funds increases until the first significant treatments are reported, which in turn may start the hype all over again.[24, 25] Where Regenerative Medicine currently resides in the cycle is under debate, but it is clear that hypes should be avoided at all costs.[26] Low-hanging fruit has been identified within the Regenerative Medicine community (more specifically; reconstructive medicine), where acellular biomaterials have been developed and used for the treatment of e.g., hernia's, burns, ulcers and heart valve anomalies.[27] These acellular biomaterials currently hold a large portion of the market and are generating useful long-term clinically relevant data and revenue for the further development of other more sophisticated treatments. Although currently viewed as a cliché, the

well-known proverb derived from one of Aesop's fables; "The Tortoise and the Hare", is very suiting to the current state of Regenerative Medicine. In our view, "slow and steady wins the race" is highly applicable, where small steps and seemingly slow progress will lead to the sustainable growth of the Regenerative Medicine community and avoid following in the footsteps of gene therapy. In this day and age, despite the current information technology infrastructure facilitating global collaboration, cooperation between academia and industry will prove to be of utmost importance in controlling the public's expectations. Creating an open environment where academia and industry can diligently work on realistic Regenerative Medicine-based solutions.[28]

The design of biomaterials will become more important with respect to the emergence of personalized medicine.[29] Each tissue's ECM has a unique composition and the latter will also apply to the scaffold that is optimal for that specific tissue.[30] Each scaffold should be fine-tuned to the specific application it is being used for.[31] It is inevitable that the complexity of scaffolds will increase, however, the success of future treatment modalities lie within their simplicity.[7] We believe that fellow researchers should focus, wherever possible, on designing products that can be used off-the-shelf and are preferably cell-culture free. Totally cell-free options should enjoy preference in order to lower costs, circumvent error-prone steps and simplify regulatory issues. Cell-free treatment modalities heavily lean on the effectivity of the scaffold in question. Many applications, predominantly larger tissues, require a form of cellularization before it can be considered as a functional replacement.

Luckily, techniques like omentum wrapping or using the patient's body as a bioreactor can hold the key to success for efficacious application of the envisioned treatment strategy.[32] Currently, omental wrapping is frequently applied at the time of tissue reconstruction.[33] The omentum is then merely used as a possible supply vasculature.[34] In this case the biomaterial is required to be directly functional as an replacement tissue. Omentum wrapping and a considerable pre-incubation time leads to a suitable vasculature and may also decrease the possibility of an adverse reaction of the body.[14] Logic dictates that by implanting scaffolds directly into the omentum prevents direct contact with a major incision, which normally leads to an inflammatory response. Therefore, omental pre-incubation should give the body time to gently react to the scaffold and subsequently start the infiltration of cells and vasculature. It is conceivable that the body will react more mildly to a construct that has been partially resorbed than a fresh non-incubated scaffold.

Next to omental wrapping, other techniques exist which do not need error-prone cell isolation and lengthy culturing steps.[35] Techniques like isolation of platelet rich plasma and sequestration of adipose derived stem cells increases the amount of straightforward options that the surgeons have to increase the affectivity of collagen-based treatment modalities.[36, 37] Commercially available systems to isolate autologous platelet rich plasma readily exist, and can be used directly as an adjuvant of an existing therapy.[38] The isolation of adipose derived stem cells from autologous fat is becoming increasing standardized and has readily found its way into cosmetic surgery.[39] To conclude, the combination of these systems which harness the power of autologous ingredients will prove to be an essential adjuvant to the next generation collagen-based scaffolds.

SAMENVATTING

Regeneratieve geneeskunde is een nieuwe, snel groeiende discipline in de medische wetenschap.[1] Een van de belangrijkste strategieën in de regeneratieve geneeskunde is gebaseerd op stimulering van natuurlijke genezingsmechanismes van het lichaam door in beschadigde weefsel materialen te implanteren (met of zonder cellen) die de extracellulaire matrix (ECM) nabootsen.[2] In de eerste fase van regeneratie fungeert de ECM hoofdzakelijk als een steiger waaraan de cellen zich kunnen hechten, naartoe kunnen migreren, en waarin ze in staat zijn om zich te vermeerderen en differentiëren. Idealiter verhindert dit de vorming van littekenweefsel en stimuleert dit vervolgens de vorming van nieuw en tevens functioneel weefsel, zodat regeneratie van het weefsel/orgaan plaatsvindt.[3] Materialen die de ECM nabootsen, ook wel 'biomatrices' of 'scaffolds' genoemd, kunnen in twee groepen worden verdeeld: scaffolds bestaand uit gedecellulariseerde weefsels[4, 5] of scaffolds gemaakt van moleculair gedefinieerde ingredienten van natuurlijke en/of synthetische afkomst.[5, 6] Afhankelijk van het doelweefsel of -orgaan, dient de scaffold te voldoen aan verschillende biologische en mechanische vereisten.

In **hoofdstuk 1** wordt een uitgebreid overzicht gegeven van de verschillende strategieën, materialen en methodologieën die in de weefseltechnologie en de regeneratieve geneeskunde worden gebruikt. Vervolgens wordt in **hoofdstuk 2** de huidige stand van de weefseltechnologie en de regeneratieve geneeskunde besproken met betrekking tot het ademhalingsstelsel (inclusief de longen, het tracheobronchiaal systeem en het diafragma). Na kritisch onderzoek van de meest recente, kwalitatief hoogstaande vakliteratuur op dit gebied is de conclusie dat, ondanks de vele veelbelovende uitkomsten van allerlei haalbaarheidsstudies, er nog een lange weg te gaan is, met veel voorziene en onvoorziene valkuilen.

In het algemeen, van de meest binnen het bereik liggende toepassingen van weefseltechnologie en regeneratieve geneeskunde, hangt het succes vaak af van de 'eenvoud' van het doelweefsel. Bij weefsels met hoofdzakelijk een mechanische functie lijkt de benadering veel eenvoudiger te zijn. Zoals bij veel behandelmethoden wordt het succes van een nieuwe techniek vaak bepaald door de eenvoud ervan.[7] Doelweefsels met hoofdzakelijk een mechanische functie zijn bijvoorbeeld bot- en kraakbeenweefsels en ligamenten. Holle organen zoals de luchtpijp, slokdarm, grotere bloedvaten en lagere urinewegen hebben echter eveneens overwegende mechanische functies.[8] Het doel van deze dissertatie was nieuwe technieken te ontwikkelen en bestaande procedures aan te passen om zo bij te dragen aan de uitbreiding van het momenteel beschikbare scaffold-instrumentarium voor het repareren van defecten in grote, holle organen.

POREUZE, OP COLLAGEEN-GEBASEERDE, BUISVORMIGE SCAFFOLDS

Poreuze scaffolds kunnen worden geconstrueerd van veel verschillende materialen. In deze dissertatie hebben wij gefocussed op gebruik van gezuiverd onoplosbaar runder type I collageen (hierna: "collageen"). Poreuze scaffolds worden gemaakt door collageen te zwelling in zuur en verolgens te homogeniseren, gecontroleerd te bevriezen en vervolgens te vriesdrogen.[9] Het resultaat van dit proces is een microporeuze scaffold van collageen dat voorheen succesvol is gebleken als ECM-nabootsend materiaal ter stimulering van de *in-vivo*-regeneratie van weefseldelen.[10, 11] Door wijziging van de matrijs waarin het vormgevings- en vriesproces plaatsvindt, kunnen buisvormige varianten van de scaffolds worden geproduceerd.[12] De studies die tot op heden zijn verricht betroffen in het algemeen hoofdzakelijk toepassingen met een kleine diameter in bijvoorbeeld bloedvaten en de lagere urinewegen.[13] Voor toepassingen met een grotere diameter (>ø15 mm) werd het proces opnieuw bekeken en geoptimaliseerd. In **hoofdstuk 3** wordt het proces beschreven waarbij buisvormige, op collageen-gebaseerde scaffolds met een grote diameter werden ontworpen voor eventueel toekomstig gebruik ten behoeve van de regeneratie van de slokdarm.[14] Om eventuele problemen met grotere diameters te ondervangen werd daarnaast gepoogd de mechanische eigenschappen aan te passen door toevoeging van een extra laag collageen of een matje van synthetische polymeer (polycaprolacton). Omdat door het ontbreken van een geschikte vasculatuur in grotere structuren

de behandelmethoden vaak geen succes bleken, werden in een schaapmodel de bovengenoemde scaffolds ingepakt in een stukje vetschort (omentale pre-implantatie) ten einde de prevascularisatie van de structuren te bevorderen. Na een incubatietijd van 6 weken toonde immunohistochemische evaluatie aan dat overal in de scaffolds zich nieuwe bloedvaten hadden gevormd. Bovendien was er vrijwel geen sprake van ontstekingen of verkalking. Dit geeft aan dat buisvormige scaffolds met een grote diameter in combinatie met omentale pre-implantatie een waardevolle toevoeging kunnen vormen aan het instrumentarium in de weefseltechnologie en regeneratieve geneeskunde.

De ontwikkeling van collageen scaffolds met een grote diameter wordt verder beschreven in **hoofdstuk 4**, waarin het proces opnieuw werd onderzocht ten einde een op collageen en een polymeer gebaseerde hybride scaffold met een behoorlijke lengte te kunnen produceren voor gebruik bij de aanleg van een urinestoma.[15] Een commercieel verkrijgbaar matje van polymeer (VYPRO II Mesh) werd geïntegreerd in een buisvormig collageen scaffold met een lengte van 12 cm, luminale diameter van 15 mm en een wanddikte van 6 mm. De scaffolds werden gesteriliseerd en geïmplanteerd als een incontinent urostoma in een varkensmodel, waarbij alleen de rechterurineleider werd gebruikt en de linkerurineleider bleef aangesloten op de blaas. De resultaten waren bemoedigend, maar niet geheel positief. In de meeste gevallen functioneerde de scaffold goed als een incontinent urostoma met neovascularisatie van de structuur en re-urothelialisatie van het lumen. Het semi-absorbeerbare synthetische matje (bestaande uit een combinatie van VICRYL en PROLENE) bleek echter ongewenste biologische reacties te veroorzaken. Bij de matjes vond afzetting van fibroblasten plaats en dit leidde vervolgens tot contractie van het weefsel. In **hoofdstuk 3 en 4** wordt het potentieel van een op collageen en polymeer gebaseerde hybride scaffold gedemonstreerd en wordt daarnaast het belang van het gebruik van het juiste polymeertype voor elke toepassing benadrukt.

De toevoeging van andere natuurlijke of synthetische polymeren is een logische methode, die vaak wordt gekozen voor het wijzigen van de mechanische eigenschappen van op een collageen-gebaseerde scaffold. Methoden gebaseerd op wijziging van de eigenschappen van het collageen zelf zijn echter beperkt. Crosslinking met carbodiimiden is een populaire techniek om collageenscaffolds te versterken. Ondanks het veelvuldig gebruik ervan in combinatie met collageen is het potentieel van crosslinking met carbodiimiden nog niet volledig gerealiseerd. In **hoofdstuk 3 en 6** wordt een nieuwe methode beschreven waarmee de mogelijkheden om de mechanische eigenschappen van collageen te wijzigen aanzienlijk worden uitgebreid. Door de poreuze collageenscaffolds gedurende crosslinking met carbodiimiden op verschillende manieren samen te drukken, kunnen de mechanische eigenschappen van de scaffolds zodanig worden gewijzigd dat de scaffolds ofwel stijver ofwel elastischer worden. In **hoofdstuk 6** wordt beschreven hoe de wand van een poreuze, buisvormige scaffold werd gecomprimeerd tot ongeveer 10 % van de oorspronkelijke dikte en vervolgens werd geplooid voordat crosslinking met carbodiimide plaatsvond. Hierdoor ontstond een collageenscaffold met bepaalde elastische eigenschappen. De comprimering van de wanddikte op zich leidde tot grotere dichtheid van het materiaal en daardoor tot grotere stijfheid van de scaffold. Door de plooiing ontstonden er microscopisch kleine vouwen in de wand van de scaffold, die werden gefixeerd tijdens het proces van crosslinking. Omdat de collageenwand een geplooide structuur heeft kan hij zonder schade worden uitgevouwen. Als de uitvouwende kracht wordt opgeheven, neemt de geplooide scaffold zijn oorspronkelijke vorm weer aan. Dit elastisch gedrag is nog nooit eerder waargenomen in collageenscaffolds. Aldus werd aangetoond dat een combinatie van fysieke druk en crosslinking kan leiden tot nieuwe mechanische eigenschappen.

NIEUWE TECHNIEKEN VOOR HET PRODUCEREN VAN GROTE HOLLE SCAFFOLDS

Om grote holle organen zoals de urineblaas te kunnen nabootsen, werden de bestaande bevriezings- en vriesdroogtechnieken aangepast. In **hoofdstuk 7** wordt de ontwikkeling van een bolvormige, holle scaffold voor de toekomstige ontwikkeling van een neoblaas beschreven. Een op maat gemaakte mal werd ontworpen om de grootte en vorm van een blaas na te bootsen. De mal werd ook zo ontworpen dat anastomose-locaties voor de urineleiders en urinebuis zorgen voor een naadloze ïntegratie. Vervolgens werd een techniek ontworpen waarmee de scaffold in een enkele stap kan worden gegoten, waardoor, na het vriesdrogen, een holle, poreuze scaffold ontstaat. De morfologische eigenschappen van de scaffold werden gekarakteriseerd. De scaffold had een gesloten lumen en in dezelfde richting georiënteerde poriestructuren (van

de buitenkant naar het midden van de bol). De cytocompatibiliteit werd eveneens getest door cultivering van varkens-urotheel en -gladdespiercellen op de scaffold. De cellen die zich hechtten aan en zich vermeerderden op de blaasscaffold behielden hun fenotype zoals immunohistochemisch werd aangetoond. De techniek voor het maken van reproduceerbare, naadloze, holle collageen-gebaseerde scaffolds zou de eerste stap kunnen zijn voor de ontwikkeling van een kant-en-klaar product voor de engineering van neoblazen.

Methoden voor het produceren van holle scaffolds zijn hoofdzakelijk gebaseerd op de eerder genoemde bevriezings- en vriesdroogtechnieken. In **hoofdstuk 5** wordt een nieuwe methode beschreven waarmee buisvormige scaffolds met gecontroleerde uitlijning van collageenvezels werden geëxtrudeerd met gebruik van een extrusiemachine met in tegengestelde richting draaiende conussen.[16] Een hoog viscueze collageensuspensie (4% g/v) werd geëxtrudeerd met constante snelheid op een spil van 25 mm. Tijdens de extrusie werd een in tegengestelde richting draaiende kracht uitgeoefend op het collageen. Met behulp van multifotonmicroscopie konden de collageenvezels driedimensionaal worden gevisualiseerd zonder gebruik van kleuring of andere verwerkingstechnieken. Er werd een duidelijke correlatie gevonden waarbij de richting en mate van uitlijning van de collageenvezels gedurende extrusie een functie waren van de schuifkrachten die werden veroorzaakt door een combinatie van conusrotatie en stromingsrichting. Een gestage verandering van de vezelrichting werd waargenomen in alle delen van de scaffold. Met deze nieuwe technologie, die in hoge mate aanpasbaar is, worden gecontroleerd buisvormige collageenscaffolds geproduceerd, waarbij de productie gemakkelijk kan worden opgeschaald.

ALGEMENE CONCLUSIE

Onoplosbaar type I collageen is een zeer veelzijdig biomateriaal dat geschikt is voor gebruik bij diverse methoden. Zoals beschreven in deze dissertatie kunnen de grenzen van collageen-gebaseerde scaffolds worden opgerekt, kan de sterkte ervan worden gewijzigd door toevoeging van een synthetische polymeer, kunnen elastische eigenschappen worden geinduceerd door middel van crosslinking, kan

de vezelarchitectuur worden gewijzigd met gebruik van schuifkrachten, en kunnen in een enkele stap naadloze, holle bolvormen worden gecreëerd met gebruik van een elegante giettechniek. Modificatie van collageen-gebaseerde scaffolds kan een belangrijke rol spelen bij de afstemming van scaffolds op allerlei methoden voor de behandeling van defecten in holle organen.

TOEKOMSTVISIE

Collageen is en blijft waarschijnlijk het belangrijkste en meest veelzijdige natuurlijke biomateriaal. Het feit dat collageen alomtegenwoordig is in de ECM maakt dit materiaal de ideale kandidaat voor allerlei strategieën in de weefseltechnologie en regeneratieve geneeskunde.[17, 18] Op grond van het werk dat in deze dissertatie wordt gepresenteerd mag duidelijk zijn dat collageen dient te worden beschouwd als de basis voor veel regeneratieve geneeskunde strategieën. In een zeer uitvoerig overzicht dat onlangs werd gepubliceerd werd de huidige stand van de regeneratieve geneeskunde met betrekking tot de behandeling van slokdarmdefecten besproken.[19] Zoals in veel overzichten van de weefseltechnologie en regeneratieve geneeskunde kwamen de auteurs tot de conclusie dat verder onderzoek nodig is. Het feit dat collageen werd gebruikt in veel van de vermelde pogingen en strategieën verbaasde ons echter geenszins en sterkte ons in de overtuiging dat collageen in belangrijke mate het succes van toekomstige strategieën zal bepalen.

De biocompatibiliteit van collageen in de categorie van natuurlijke biomaterialen is ongeëvenaard. Echter het probleem bij componenten verkregen uit dierlijk materiaal is dat deze overdraagbare pathogene micro-organismen kunnen bevatten zoals virussen, bacteriën en prionen. Momenteel wordt het meeste collageen voor medisch gebruik verkregen uit runderen, varkens en schapen en meestal van dieren die in de beslotenheid van een kudde zijn opgegroeid.[20] Ondanks de grote hoeveelheid collageen dat als bijproduct van de vleesindustrie wordt geproduceerd, is het toch zeer wel mogelijk dat op basis van gecontroleerde hoefdierbronnen moeilijk aan de groeiende vraag naar gecertificeerd collageen voor medisch gebruik zal kunnen worden voldaan.

De mogelijkheid om andere ECM-componenten uit gestandaardiseerde dierlijke bronnen te isoleren dient eveneens te worden onderzocht. Een grotere inspanning is nodig om andere moleculair gedefinieerde componenten of modificatiemethoden te vinden die met collageen een synergistisch effect opleveren bij wondgenezing en weefselregeneratie. Waarschijnlijk vraagt elke doeltoepassing om een specifiek "recept" of een unieke combinatie van collageen met andere ECM-gebaseerde componenten zoals glycosaminoglycanen, elastine, componenten van het basaalmembraan, en groeifactoren.[21] We denken dat de beschikbaarheid van meer medisch gecertificeerd ECM- componenten zou kunnen leiden tot meer gericht onderzoek. Vaak blijven onderzoeksgroepen momenteel steken in de ontwikkelingsfase, blijkbaar als gevolg van het gebrek aan medische certificering van ECM-gebaseerde biomaterialen. Als verschillende onderzoeksgroepen gaan samenwerken en daarbij gestandaardiseerde, commercieel verkrijgbare, medisch gekwalificeerde materialen gebruiken, kan de (pre)klinische onderzoeksbarrière misschien gemakkelijker worden geslecht.

Hoewel er nog veel werk moet worden verzet om effectieve biomaterialen en behandelmethoden (zowel acellulaire als op celgebaseerde) te vinden, is de huidige markt voor regeneratieve geneeskunde nu al goed voor een jaarlijkse omzet van enkele miljarden dollars.[22] Hopelijk heeft de wereld van de regeneratieve geneeskunde lering getrokken uit de fouten die in de jaren tachtig en negentig van de vorige eeuw zijn gemaakt tijdens de hype rond de gentherapie, want nu, decennia later, komen immers pas de eerste goedgekeurde behandelingen met gentherapie beschikbaar.[23] Het is niet ongewoon dat een nieuwe technologie te maken krijgt met de "Gartner Hype Cycle", waarbij een veelbelovende doorbraak leidt tot overdreven vooruitzichten en beloften totdat duidelijk wordt dat alles veel meer tijd, geld en werk vergt. Hierdoor raakt het grote publiek teleurgesteld en wordt het vervolgens moeilijk de benodigde onderzoeksgelden te vergaren, dat wil zeggen totdat de eerste significante behandelingsresultaten worden gepubliceerd, waarna de hype weer helemaal opnieuw begint.[24, 25] Waar de regeneratieve geneeskunde zich nu precies in deze cyclus bevindt is niet geheel duidelijk, maar wel duidelijk is dat hypes te allen tijde dienen te worden vermeden.[26] Waar precies makkelijke successen zijn te behalen is reeds bekend in de regeneratieve geneeskunde (meer in het bijzonder in de reconstructieve geneeskunde). De ontwikkelde acellulaire biomaterialen worden o.a. gebruikt voor de behandeling van liesbreuken, brandwonden, zweren en hartklepafwijkingen.[27] Deze acellulaire biomaterialen beheersen thans een groot deel van de markt en genereren nuttige en klinisch relevante gegevens voor de lange termijn evenals inkomsten die kunnen worden aangewend voor de verdere ontwikkeling van andere, meer geavanceerde behandelingen. Ook al is het een cliché, het bekende spreekwoord "haastige spoed is zelden goed" dat is afgeleid van de fabel van Aesopus over de haas en de schildpad is zeer van toepassing op de wereld van de regeneratieve geneeskunde, waarin met kleine stappen en schijnbaar langzame voortgang zeker duurzame groei kan worden verwezenlijkt, en zo eveneens kan worden vermeden dat men in de voetstappen treedt van de gentherapie. Ook al wordt wereldwijde samenwerking tegenwoordig vergemakkelijkt door de ICT-infrastructuur, het zal in deze huidige tijd toch nodig zijn dat universiteiten en bedrijven zich inspannen om via goede samenwerking de verwachtingen bij het grote publiek in toom te houden. Door een open omgeving te creëren waarin universiteiten en het bedrijfsleven goed kunnen werken aan realistische oplossingen gebaseerd op de regeneratieve geneeskunde kan het proces van duurzame groei worden gestimuleerd en zal men uiteindelijk belangrijke bijdragen kunnen leveren aan zowel de private als publieke sector.[28]

Het ontwerpen van geschikte biomaterialen zal belangrijker worden naar mate gepersonaliseerde geneeskunde steeds meer ingang krijgt.[29] De ECM van elk weefsel is uniek qua samenstelling en daarom dienen scaffolds daar optimaal op aan te sluiten.[30] Elke scaffold dient te worden afgestemd op de specifieke toepassing waarvoor hij wordt gebruikt.[31] Het is onvermijdelijk dat de complexiteit van scaffolds zal toenemen, maar desalniettemin ligt het succes van toekomstige behandelmethoden besloten in hun eenvoud.[7] We vinden dat, waar mogelijk, onze collega-onderzoekers zich dienen te richten op het ontwerpen van kant-en-klare producten die bij voorkeur celvrij zijn. Volledig celvrije opties dienen de voorkeur te hebben opdat de kosten kunnen worden teruggebracht, foutgevoelige stappen kunnen worden omzeild en de regelgeving kan worden vereenvoudigd. Celvrije behandelmethoden zijn sterk afhankelijk van de effectiviteit van de betreffende scaffolds. Voor veel toepassingen, vooral bij grotere weefsels, is eerst een vorm van cellularisatie nodig voordat ze kunnen worden beschouwd als een functionele vervanging.

Gelukkig kunnen technieken zoals omentale pre-implantatie en het gebruik van het lichaam van de patiënt als bioreactor, de sleutel tot succes vormen voor een doeltreffende toepassing van de beoogde behandelstrategie.[32] Tegenwoordig wordt

omentale pre-implantatie vaak gebruikt voor weefselreconstructie.[33] Het stukje buikweefsel wordt dan slechts gebruikt als mogelijke bron voor de creatie van nieuwe vasculatuur.[34] Omentale pre-implantatie en een lange pre-incubatietijd resulteren in een geschikte vasculatuur en kunnen eveneens het risico van afstoting door het lichaam verminderen.[14] Door scaffolds direct in het buikweefsel te implanteren is een grote incisie (die gebruikelijk een ontstekingsreactie veroorzaakt) overbodig. Omentale pre-incubatie geeft het lichaam de tijd om te reageren op de scaffold en deze vervolgens te infiltreren met cellen en bloedvaten. Het is niet ondenkbeeldig dat het lichaam milder zal reageren op een structuur die gedeeltelijk is geresorbeerd dan op een verse niet-geincubeerde scaffold.

Naast omentale pre-implantatie bestaan er andere technieken waarvoor geen foutgevoelige cel-isolatie en tijdrovende kweekstappen nodig zijn.[35] Technieken zoals isolatie van plaatjesrijk plasma en sekwestratie van stamcellen uit vetweefsel, vergroten de mogelijkheden voor chirurgen om op redelijk eenvoudige wijze de effectiviteit van op collageen-gebaseerde behandelmethoden te verbeteren.[36, 37] Systemen voor het isoleren van lichaamseigen, plaatjesrijk plasma zijn commercieel verkrijgbaar en kunnen direct worden gebruikt ter ondersteuning van een bestaande therapie.[38] Isolatie van uit lichaamseigen vetweefsel verkregen stamcellen wordt steeds meer gestandaardiseerd en heeft ruim ingang gevonden in de cosmetische chirurgie.[39] De combinatie van deze systemen die de gebruikmaken van de kracht van lichaamseigen componenten zal in belangrijke mate bijdragen aan de ontwikkeling van de volgende generatie collageen-gebaseerde scaffolds.

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CHAPTER 09

CURRICULUM VITAE, LIST OF PUBLICATIONS AND ACKNOWLEDGEMENTS

CURRICULUM VITAE

Henk Hoogenkamp werd geboren op 1 april 1987 te Douglasville (GA, Verenigde Staten). In 2004 behaalde hij zijn HAVO diploma waarna hij aan de opleiding Life Science aan de hogere laboratorium opleiding (HLO, Nijmegen, Nederland) begon. Tijdens het 3e jaar van zijn studie liep Henk in stage bij Best Provision Co. Inc. (Newark, NJ, Verenigde Staten) waar hij heeft gewerkt aan het gebruik van bacteriofagen ten behoeve van voedselveiligheid in de vlees industrie. Zijn afstudeerstage vond plaats op het Radboud Universiteit Medical Center (Radboudumc, Nijmegen, Nederland). Hier werd hij geplaatst bij het Europees consortium project; EuroSTEC (Soft tissue engineering for congenital birth defects in children). Afdelingen Urologie en Biochemie van het Radboudumc speelden een sleutelrol in het Tissue Engineering project, waarbij Henk de effecten van sterilisatie van op collageen gebaseerde biomaterialen in kaart bracht. Na succesvolle afronding van zijn beide stages, behaalde Henk in 2008 zijn Bachelor of Applied Lifescience. Aansluitend startte Henk gelijk zijn vervolgopleiding Molecular Lifesciences waarbij hij zijn werkzaamheden aan de Radboudumc voortzette, maar ditmaal als parttime research technician. Eind 2009 behaalde hij zijn Master of Applied Lifescience. Hierna, startte Henk als researcher bij Marel Townsend Further Processing waarbij hij zijn ervaring met collageen toe kon passen op het gebeid van co-extrusie van vleesproducten.

Tijdens zijn werkzaamheden bij Marel, zag Henk veel overeenkomsten met de vraagstukken die zich afspeelde bij het Radboudumc op het gebied van Tissue Engineering en Regenerative Medicine. Hierbij besloot hij de twee partijen samen te brengen en gemeenschappelijke interesses in kaart brengen. Uiteindelijk, werd er voldoende basis gevonden om een gezamenlijk project te starten waarbij Henk deeltijd aan zijn promotie onderzoek aan de Radboudumc kon werken en tegelijkertijd Marel kon blijven ondersteunen bij verschillende projecten onder directe begeleiding van Dr. Toin van Kuppevelt en Dr. Willeke Daamen. Dit proefschrift beschrijft het resultaat van zijn periode bij het Radboudumc. Henk heeft meerdere internationale beurzen en congressen/symposia bijgewoond en bij verschillende zijn werk gepresenteerd waaronder de EuroSTEC consortium meetings (2008 Uppsala Zweden, 2009 Groningen Nederland, 2009 Leuven België, 2010 Amsterdam Nederland), TERMIS congressen (2011 Grenada Spanje, 2014 Atlanta Verenigde Staten), voedingsbeurzen (2010/2013 IFFA Frankfurt Duitsland, 2011 FIC Shanghai China, 2013 IFT Chicago Verenigde Staten) en vakspecialistische bijeenkomsten (2012 Collagen symposium Freiberg Duitsland, 2009 ESPU Amsterdam Nederland). Eind 2014 is zijn promotie traject tot een einde gekomen. In december van 2015 mag hij zijn proefschrift verdedigen.

LIST OF PUBLICATIONS

1. Collagen fiber alignment: Directing collagen fibers using counter-rotating cone extrusion. *Henk Hoogenkamp, Gert-Jan Bakker, Louis Wolf, Patricia Suurs, Bertus Dunnewind, Shai Barbut, Peter Friedl, Toin H. van Kuppevelt* and *Willeke F. Daamen.* Acta Biomaterialia, Vol. 12 (2015), pp. 113-121

2. Seamless vascularized large-diameter for esophageal Regenerative Medicine: Tubular collagen scaffolds reinforced with polymer knittings. *Henk Hoogenkamp, Martin Koens, Paul Geutjes, Herwig Ainoedhofer, Geert Wanten, Dorien Tiemessen, Jöns Hilborn, Bhuvanesh Gupta, Wouter Feitz, Willeke F. Daamen, Amulya Saxena, Egbert Oosterwijk* and *Toin H. van Kuppevelt*. Tissue Engineering Part C: Methods, Vol. 20, No. 5 (2014), pp. 423-430

3. Vicryl scaffolds for reconstruction of the diaphragm in a large animal model. *Katrien Brouwer, Willeke F. Daamen, Henk Hoogenkamp, Paul Geutjes, Ivo Blauw, Wilma Janssen-Kessels, Willem Boode, Elly Versteeg, René Wijnen, Wout Feitz, Marc Wijnen* and *Toin H. van kuppevelt*. Journal of Biomedical Materials Research Part B: Applied Biomaterials, Vol. 102 No. 4 (2014), pp. 756-763

4. Regenerative Medicine for the respiratory system: Distant future or tomorrow's treatment? *Katrien Brouwer, Henk Hoogenkamp, Willeke F. Daamen* and *Toin H. van Kuppevelt.* American Journal of Respiratory and Critical Care Medicine, Vol. 187, No. 5 (2013), pp. 468-475

5. Tissue engineered tubular construct: Urinary diversion in a pre-clinical porcine model. *Paul Geutjes, Luc Roelofs, Henk Hoogenkamp, Mariëlle Walraven, Barbara Kortmann, Robert de Gier, Fawzy Farag, Dorien Tiemessen, Marije Sloff, Egbert Oosterwijk, Toin H. van Kuppevelt, Willeke F. Daamen* and *Wout Feitz.* The Journal of Urology. Vol. 188, No. 2 (2012), pp. 653–660

6. *In Vitro* Evaluation of Type I Collagen-Based Scaffolds After Applying Different Sterilization Techniques. *Henk Hoogenkamp, Dorien Tiemessen, Keauis Faraj, Willeke F. Daamen, Toin H. van Kuppevelt, Egbert Oosterwijk, Paul Geutjes* and *Wout Feitz.* Journal of Pediatric Urology Vol. 5 (2009) S19

7. Beyond the future of lab grown hamburgers. *Henk W. Hoogenkamp* and *Henk Hoogenkamp.* Fleischwirtschaft international, Vol. 6 (2013), pp. 25-26

8. Your enemy's foe is your friend. *Henk Hoogenkamp.* Fleischwirtschaft International Vol. 2 (2009) pp. 44-49

PATENTS

1. Method of extruding sausage sleeves of at least partial collagen, extrusion head and extruded collagen sleeves. *Albertus Dunnewind, Patricia Rosa Maria Hoekstra-Suurs, Wilhelmus Johannes Everardus Maria van den Dungen, Johannes Martinus Meulendijks, Frank Johannes Antonius van den Heuij* and *Henk Robert Hoogenkamp*. WO 2015034355 A1 (2015).

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Henk Hoogenkamp
2015

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